Metabolomics – an analytical strategy for identification of toxic mechanism of action

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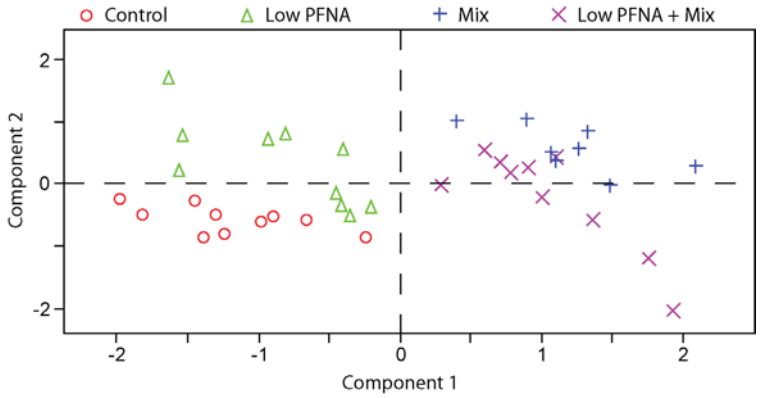

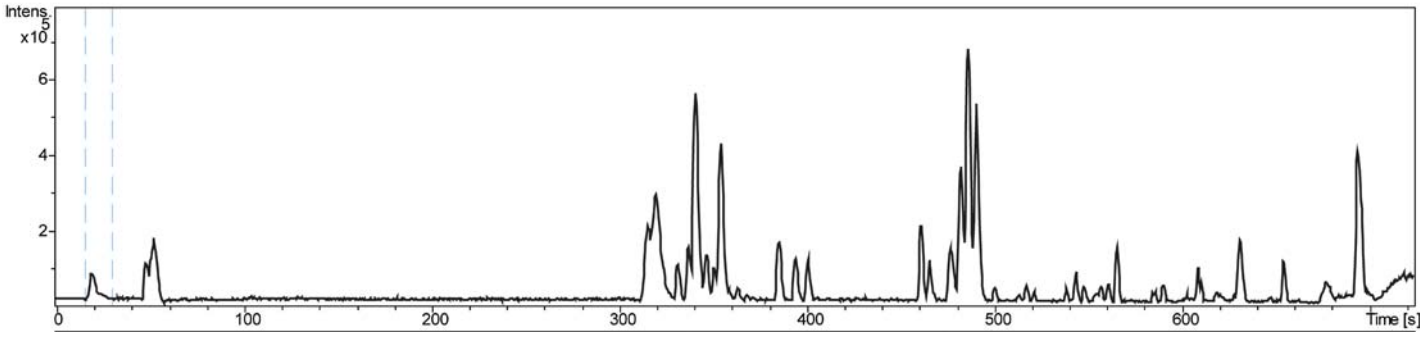

Kasper Skov
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DTU Food
National Food Institute

Summary

Humans are exposed to chemicals from diverse sources such as foods, pharmaceuticals, cosmetics and the air (Monosson 2005), which may affect human health, even causing serious disease or death (Nielsen et al 2010a). Toxicology is concerned with the study of toxic effects exerted by chemicals on a living organism, but also associated to issues related to poisons, being it clinical, industrial, or legal.

Metabolism is the set of chemical reactions that allow an organism to maintain its functions, whereas metabolites are its intermediates and products. The complete set of small molecule metabolites is referred to as the metabolome and the comprehensive and quantitative analysis of all metabolites is denoted metabolomics (Fiehn 2001). The effects of toxic compounds as physiological or chemical induced changes in the mammalian body are reflected in the plasma metabolome.

In toxicology, compounds with an effect on hormone regulation have attracted much attention. These are the so-called endocrine disrupters which mimic natural endogenous hormones and are suspected to be involved in the observed decreased fertility in Denmark over the last two decades. Since endocrine disrupting chemicals (EDCs) have effects on humans at low concentration, one of the present challenges in toxicology is to develop methods capable of measuring exposures and effects at the low end of the scale.

The aim of the present work was to scrutinize the impact toxic chemicals can have on the metabolome, in particular EDCs at low, human relevant concentrations. In order to accomplish this, a sample preparation technique suited to handle a wide range of metabolites in the plasma metabolome was developed. The technique included sub-fractionation by solid phase extraction into three sub-samples. The resulting method showed approximately 2.5 times more molecular features compared to that obtained if only protein precipitation was applied. The performance of the method was investigated using plasma samples from rats administered to the environmental pollutant perfluorononanoic acid (PFNA) and was applied in the metabolome analyses throughout the thesis.

Humans are simultaneously exposed to multiple chemicals, many of which can be detected in human body fluids; however, the consequences of low dose exposure to complex mixtures of chemicals are poorly understood. By use of two omics approaches, metabolomics and transcriptomics, the effects on rats caused by exposure to a 14-compound mixture (Mix) ± PFNA were profiled. The applied technologies provided complementary information allowing for a detailed analysis of the affected signalling pathways. Mix alone caused reduced lipid concentration evident in plasma. The hepatic effects on lipid metabolism were mainly driven by PFNA by activation of the PPAR receptors. This study verifies that a chemical mixture given at

high-end human exposure levels can affect lipid homeostasis. In a follow-up study on the importance of exposure to complex real-world mixtures, data suggested that mixtures of environmental chemicals at doses approaching high-end human exposure levels can cause a hormonal imbalance, with increased plasma corticosterone levels, and disturb steroid hormones and their regulation.

To evaluate effects caused by low-dose exposure, data from the PFNA study was used alongside data from a bisphenol A study. Plasma from pregnant rats and their offspring exposed to bisphenol A at an exposure concentration of 25 and 250 µg/kg bw/day were analyzed. A decrease in monoacylglycerol(18:0) and monoacylglycerol(16:0) in the mother animals was observed, while for the male offspring, increase in lyso-phosphotidylcholine plasma concentration was observed.

The developed platform detects three main groups of metabolites; a phospholipid fraction, a lipid fraction and a polar fraction and reveals changes in the metabolome that could not be foreseen using regular toxicological approaches. The study presents a new approach which improves the basic biochemical understanding of toxic exposures to the rat. From this data it is feasible not only to determine the endpoint of the toxic exposure, but to suggest biochemical precursors to the exposure.

Resumé (In Danish)

Mennesket er eksponeret for kemikalier fra mange forskellige kilder, blandt andet mad, medikamenter, kosmetik og atmosfærisk luft (Monosson 2005). Mange af disse kemikalier kan påvirke menneskets helbred, i nogle tilfælde påføre store skader og i værste fald slå folk ihjel (Nielsen et al 2010a). Toksikologien er den videnskab der beskæftiger sig med studiet af kemikaliers toksiske effekter på den levende organisme. Den er også associeret til spørgsmål relateret til giftstoffer, vedrørende kliniske, industrielle eller juridiske problemer.

Metabolisme er det sæt af kemiske reaktioner som er essentielle for at opretholde organismens funktioner, mens metaboliter er dens intermediater og produkter. Den samlede mængde af metaboliter benævnes metabolomet og den omfattende og kvantitative analyse af alle metaboliter kaldes metabolomics (Fiehn 2001). Ændringer forårsaget af stoffer der fysiologisk eller kemisk påvirker den menneskelige krop kan observeres i plasma metabolomet.

Stoffer, der påvirker hormonreguleringen, har fået stor opmærksomhed i toksikologien. Disse stoffer er såkaldte hormonforstyrrende stoffer, som efterligner de naturlige hormoner. I Danmark er der observeret en nedsat fertilitet over de sidste to årtier, og det menes at de hormonforstyrrende stoffer kan være medvirkende til denne nedgang. Eftersom de hormonforstyrrende stoffer har effekt på mennesket ved lav koncentration er en af de nuværende udfordringer i toksikologien, at udvikle metoder til måling af ændringer ved eksponering ved lave koncentrationer.

Formålet med denne afhandling var at undersøge påvirkningen af toksiske kemikalier på metabolomet, især med fokus på de hormonforstyrrende stoffer ved lav human relevant koncentration.

For at kunne opfylde dette formål, blev en procedure, som var baseret på en sub-fraktionering udført ved hjælp af solid-fase ekstraktion, udviklet til analyse af et bredt udsnit af metaboliter. Metoden kunne detektere 2,5 gange flere molekylære features end en metode der kun anvendte protein fældning, og blev testet i et rotteforsøg, hvor dyrene var eksponeret til et miljøkemikalium, perfluornonanone syre (PFNA). Denne fremgangsmåde blev anvendt i metabolom analyserne i hele denne afhandling.

Mennesket er påvirket af mange kemikalier hver dag, hvoraf nogle kan blive detekteret i kropsvæsker. Men der er mangel på forståelse for, hvordan komplekse blandinger af disse stoffer vil påvirke os. For at undersøge dette brugte vi en dels metabolomics og dels transcriptomics tilgang for at undersøge effekten af PFNA med og uden en mix af 14 relevante kemikalier. Disse to fremgangsmåder blev brugt i kombination til at analysere en mulig påvirkning af biologiske pathways. Blandingen viste effekt på fedtstofskiftet

udtrykt ved en nedgang i plasma fedtstofferne, mens effekterne på leveren primært var fra PFNA ved aktivering af PPAR receptorerne. Studiet viste således at kemikalieeksponering, ved koncentrationer relevante for mennesker, kan påvirke fedtstofmetabolismen. Derudover viste forsøget at lav dosis kan påvirke hormonniveauerne i blodet hos mennesket, ved en forøget corticosterone plasma koncentration, samt reguleringen af disse.

For yderligere at kunne evaluere lavdosiseksponering blev data fra PFNA studiet anvendt sammen med data fra et bisphenol A studie. Gravide rotter blev eksponeret til 25 og 250 µg/kg/dag, og blodet fra mødre og afkom blev analyseret. En nedgang i monoacylglycerol(16:0) og monoacylglycerol(18:0) blev observeret i mødrene, mens det mandlige afkom viste et øget niveau af lyso-phosphocholine.

Den udviklede analytiske platform detekterede således tre grupper af metaboliter; fosfolipider, lipider og en polær fraktion. Platformen viste ændringer som ikke ville være fundet ved regulære toksikologiske studier. Studierne i denne afhandling præsenterer en ny strategi til at evaluere biokemiske ændringer i rotter efter toksisk eksponering. Data fra dette studie viser, at det ikke kun er muligt at påvise toksikologiske endpoints, men muliggør også at observere biokemiske forstadier, ”biomarkører”, for toksikologiske eksponering.

Contents

Preface

This PhD thesis is submitted as partial requirements for the attainment of my PhD degree. The research has been conducted between 15th of December 2011 and 15th of December 2014. The animal studies have been carried out in the Division of Toxicology and Risk Assessment, DTU Food and the metabolic profiling was performed in Division of Food Chemistry, DTU Food. The NMR experiments have been carried out in Canada, in the Wishart Group at University of Alberta, Edmonton, Alberta, Canada.

This project would not have been possible without the many people who contributed their time and expertise. My supervisors have guided me throughout and helped me to grasp what it means to be a scientist. Therefore, a special thanks to my supervisors, Niels Hadrup, Jørn Smedsgaard and Henrik Lauritz Frandsen; thank you for your guidance, for aiding in shaping the study and for all the scientific discussions.

Thanks to the laboratory technicians for assisting in purchasing chemicals, help running of the laboratories and of course, their technical assistance. Thank you Maud Bering Andersen, Liljana Petrevska, Lis Abildgaard and Lene Gram.

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Kasper Skov

List of publications

Paper I Skov, K., Hadrup, N., Smedsgaard J., Frandsen H., 2015. LC–MS analysis of the plasma metabolome—A novel sample preparation strategy. *Journal of Chromatography B*, 978-979, pp.83–88.

Paper II Hadrup, N., Petersen, M., Skov K., Hansen, N.L., Berthelsen, L. O., Kongsbak K., Boberg, Julie, Dybdahl, M., Hass, U., Frandsen, H., Vingaard, A. M. Perfluorononanoic acid in combination with 14 chemicals exerts low-dose mixture effects in rats. Archives of Toxicology, Published online.

Paper III Skov, K., Kongsbak, K., Hadrup, N., Frandsen, H., Svingen, T., Smedsgaard, J., Audouze, K., Eklund, A. C., Vinggaard, A. M. Exposure to perfluorononanoic acid combined with a low-dose mixture of 14 human-relevant compounds disturbs energy/lipid homeostasis in rats. Metabolomics. Published online

Paper IV Skov K., Hadrup N, Axelsted M., Christiansen S., Vingaard A.M., Haas U., Frandsen H. Effect of BPA on rats and their offspring – a metabolic profiling of low dose exposure to BPA, Manuscript in progress

Paper V Hadrup N., Löschner K.[2], Skov K., Ravn-Haren G., Larsen E.H.[2], Mortensen A., Lam H.R., Frandsen H. Metabolite profiling of urine from rats dosed with Selenium nanoparticles or Selenium ions; Manuscript in progress

List of Abbreviations

ANOVA	Analysis of Variance
BPA	Bisphenol A
DG	Diacylglycerol
EDC	Endocrine Disrupting Chemical
FDR	False Discovery Rate
GC	Gas Chromatography
GD	Gestation day
HDL	High Density Lipoprotein
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Pressure Liquid Chromatography
LC	Liquid Chromatography
LDL	Low Density Lipoprotein
LOAEL	Low observed adverse effect level
Lyso-PC	Lyso-phosphatidylcholine
MG	Monoacylglycerol
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
NOAEL	No observed adverse effect level
PC	Phosphotidylcholine
PCA	Principal component analysis
PFNA	Perfluorononanoic acid
PLS	Partial Least Square
PLS-DA	Partial Least Square-Discriminant Analysis
PSU	Polysulfone
qTOF	Quadrupole Time-of-Flight
SPE	Solid Phase Extraction
SPF	Specific pathogen-free
TAG	Triacylglycerol
UHPLC	Ultra High Pressure Liquid Chromatography

Introduction

Humans are exposed to chemicals from various sources including foods, pharmaceuticals, cosmetics and the air (Monosson 2005). Such exposure can lead to toxic effects resulting in compromised health and even serious disease or death (Nielsen et al 2010a). The branch of science typically concerned with these toxic effects is 'toxicology', a broad terminology encompassing all aspects of how living organisms are adversely affected by exposure to chemicals and poisons, also extending to associated problems such as clinical, industrial, or legal aspects. The prediction of toxic effects and pre-disease determinations are challenging and often time- and resource-consuming. Therefore new and more efficient techniques than what is presently available are needed.

Although toxicology deals with the potential effects on all types of tissues and organs, in this thesis the focus is on disruptions to hormone regulation in mammals. Compounds that exert such effects are referred to as endocrine disrupters, or endocrine disrupting chemicals (EDCs) and typically mimic or interfere with the natural (endogenous) hormones (Vandenberg et al 2012). The impact of EDCs has been widely studied and implicated as causative of a broad range of reproductive dysfunction phenotypes (Skakkebaek et al 2006). As EDCs can have effects on humans at low concentrations, a particular challenge in toxicology is to develop methods to detect more subtle effects, both at the molecular and physiological levels. In fact, effects of low dose exposure can be difficult to establish, as it causes changes to hormone levels and metabolism only observed at the metabolite level and not detectable by traditional toxicological endpoints. Therefore, to better understand the real consequences of EDCs, also taking into account less adverse effects that can later contribute towards adverse health effects, new and improved technologies are necessary to monitor toxic chemicals.

Significant advances in systems biology over the last couple of decades offer unparalleled opportunities to unravel some of the complex mechanisms behind the toxic effects caused by chemical exposure. The 'omics' strategies, including genomics, transcriptomics, proteomics, metabolomics, are now routinely applied across biological disciplines, with the combination of two or more 'omics' approaches becoming increasingly more popular.

The metabolism is the set of chemical reactions that allow an organism to maintain its functions and metabolites being its intermediates and products. The complete set of small molecule metabolites is referred to as the metabolome and the comprehensive and quantitative analysis of all metabolites is denoted metabolomics (Fiehn 2001).

Analysis of metabolism can give a more mechanistic insight into how specific compounds act and affect a biological system, including the human body. High resolution mass spectrometry has become the tool of choice to study metabolism and metabolites, not least due to its very high sensitivity and potential to detect a spectrum of metabolites, but also its ability to scrutinize the impact of toxic compounds on metabolites, allowing for determination of interaction.

During the last two decades the application of metabolomics has significantly changed the strategy of metabolic studies. This change has been very much driven by developments in mass spectrometry, in particular electrospray ionization MS and affordable high resolution mass analyzers. Today the methods of choice are liquid chromatography mass spectrometry (LC-MS), gas chromatography mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) (Robertson et al 2011). Using metabolomics to study the effects on metabolism by disease or external (environmental) factors has given new insights into diseases and effects of toxic exposures in humans (Souverain et al 2004; Psychogios et al 2011). Combined with other strategies such as transcriptomics, genomics and proteomics, metabolomics has proven a powerful strategy to discover modes- and mechanisms-of-action underpinning various pathologies. Metabolomics is also relatively low-cost and fast, hence a good tool to analyze larger quantities of samples (Gomase et al 2008).

The aim of this thesis was to examine what impact EDCs can have on the metabolome, particularly at low levels of exposure relevant to real human exposures. A main objective was to develop a method capable of analyzing a wide range of compounds in the plasma metabolome. This was achieved by the development of a new sample preparation technique which resulted in acquisition of data on a larger number of analytes compared to conventional techniques. Also, the metabolomics approach was compared to a more traditional toxicology approach in order to evaluate whether the new approach performed better than traditional toxicology protocols. Furthermore, a hypothesis that the metabolomics approach could reveal better mechanistic insights, and thus a better understanding of low-dose exposure effects, a lower dose of exposure than traditional protocols was tested. For one study the systemic change caused by exposure to a toxic chemical was analyzed by transcriptomics in combination with the results obtained by the metabolomics analysis.

This thesis focused on developing an improved analytical platform for the analysis of plasma metabolites. The new method resulted in approximately 2.5-times more features than a protein precipitation approach. The method was tested on animals exposed to perfluorononanoic acid (PFNA) for 14 days to evaluate if it was capable of determining metabolic changes following low-dose mixture effects in rats. Animals were first tested by a classical toxicology approach, studying effects on organs, bodyweight and blood

parameters. Then secondly by metabolomics and transcriptomics approaches to determine if the method revealed similar results. Changes in plasma hormone levels were observed by the classical approach and also corroborated by the metabolomics analysis of the blood plasma. Furthermore, the highest concentration of PFNA-exposure caused hepatic steatosis, which could also be predicted from the metabolomics and transcriptomics data, but with the latter approach also revealing additional insights into affected regulatory pathways. To further challenge the new strategy, two more low-dose exposure experiments (mothers and pups exposed to a low dose of bisphenol A (BPA)) were analyzed. Here, we found changes in both the mother animals exposed directly to BPA and in the pups exposed via the placenta and the mother milk. Thus low-dose exposure could indeed be detected in the plasma metabolome and hence be used to reveal additional information regarding effects of toxic exposures.

Before discussing the results of the individual studies undertaken during this project, a further overview of the literature will be presented. Part I will give a general introduction to toxicology and metabolomics, including an overview of the literature on the application of metabolomics approaches in toxicology. Part II outlines the analytical methods that were applied to analyze the metabolomes. Furthermore, the analytical strategies used in metabolomics, mostly targeted and non-targeted metabolomics, will be discussed, finally including a short section describing the samples of interest and how they were prepared.

The rest of the thesis contains materials and methods, a compilation of result, a section reporting results from the individual experiments, and finally; a summary discussion on low-dose exposure effect on animals and humans.

Part I: Toxicology and metabolomics

1.1 Toxicology

Toxicology is the branch of biology concerned with the study of adverse effects of chemicals on living organisms (Nielsen et al 2010b). As mentioned above, toxicology is a science that deals with poisons and can be described as the study of changes in the physiology or metabolism as a response to a toxic chemical. The conventional toxicology approach is based on analysis of the effect of a single chemical at increasing doses to detect possible adverse effects in an organism (Monosson 2005). Within toxicology the "No Observed Adverse Effect Level" (NOAEL: the highest concentration of compound with no adverse effect) and the "Lowest Observed Adverse Effect Level" (LOAEL: the lowest concentration of a compound which shows an adverse effect) (Nielsen et al 2010b) are terms used to characterize the impact of a toxic chemical evaluated by morphological endpoints, see Figure 1. Traditional methods are based on *in vivo studies* e.g. in rats in combination with *in vitro* studies e.g. cellular studies (Klaassen 2008; Taxvig et al 2011). Toxicokinetic profiles of chemical substances are usually obtained through *in vivo* studies and these often are supplement by *in vitro* studies focusing on e.g. metabolism (Klaassen 2008). The *in vivo* experiments are conducted at different time points e.g. seven days to reveal acute effects, e.g. 90 days to reveal long term effects and chronic effects by exposing the animals for at least 180 days (Frazier 1991; Nielsen et al 2010b).

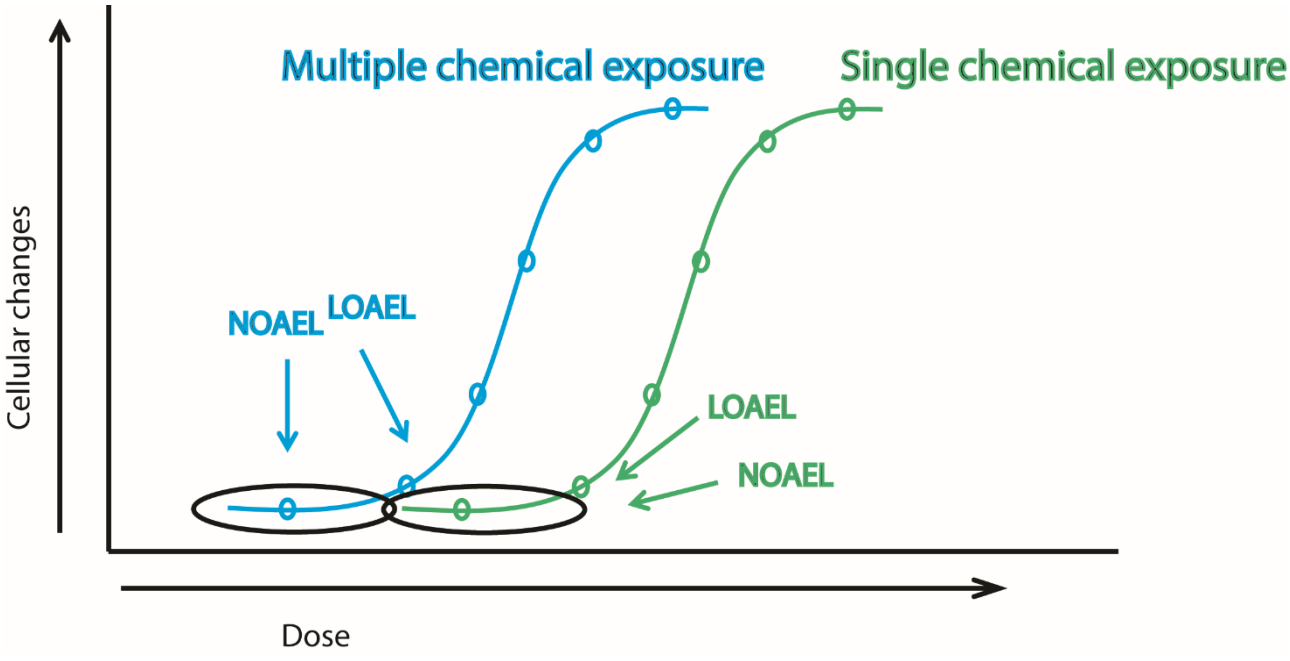

Figure 1 The effect of a single toxic chemical at different doses is illustrated in the green curve . The NOAEL and LOAEL doses are indicated. As indicated, cellular changes increase with an increasing dose. The effect of multiple chemical exposure is illustrated in the blue curve, where the animal has been treated with a dose consisting of multiple compounds resulting in a decrease in LOAEL (Nielsen et al 2010b)**.**

Conventional toxicology studies are performed at high concentrations (see Figure 1) and aims to 1) identify toxic effects, 2) identify morphological changes in the exposed organism, and 3) to establish a NOAEL.

Recently, it has been suggested that toxic compounds might interact. Figure 1 shows an example of the effect of mixtures of toxic compounds which cause an effect different from that observed from a single compound. Three main mechanisms of interaction have been suggested, namely synergism, antagonism and addition (Crofton et al 2005; Moser et al 2006; Frische et al 2009; Christiansen et al 2009). With synergism is understood that two or more compounds interact to cause an effect larger than the sum of single-compound effects; 1+1 > 2. Antagonism means that compounds counteract each other's effects so that the combined effect is reduced; 1+1 < 2. Addition is the simple scenario where the effects of the single compounds contribute towards the overall effects as mere sums of each other; 1+1 = 2 (Crofton et al 2005; Moser et al 2006; Frische et al 2009).

As mentioned above, sources of toxic chemicals are numerous, for instance air pollution, occupational exposure and food. Chemicals may include pesticides, medical residues, cardboard materials, thermal printings, plastics, surfactants and many more (Monosson 2005; Crofton et al 2005; Lau 2012). The effect exerted by these compounds can differ significantly between species, sex, doses, time of exposure, and a variety of other factors (Colborn et al 1993; Evans et al 2004). Furthermore, two individuals may react differently to the same toxic chemical due to genetic polymorphisms.

A particular group of compounds which has received much attention is EDCs (Kozlowska-Tylingo et al 2010). They are compounds that can mimic endogenous steroid hormones (Connolly et al 2011). There is evidence suggesting that EDCs affect wildlife and humans by causing reproductive, developmental, or metabolic disorders (Colborn et al 1993; Evans et al 2004; Nicolucci et al 2011; Tse et al 2013). An EDC was defined by the U.S. Environmental Protection Agency as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" (Program and Agency 2001). The potential effects of EDCs have been studied for more than two decades (Skakkebaek et al 2006) and it is believed that they can affect embryogenesis, fetal and neonatal development, as well as adult function (Colborn et al 1993).

In 2006, Skakkebaek and co-workers reported a decline in semen quality, and increase in hypospadias and testis cancer in Denmark during the last two decades, which could be associated with exposure to EDCs or other environmental chemicals (Sharpe and Skakkebaek 2003; Skakkebaek et al 2006). EDCs have been shown to affect human biology at low concentrations of exposure (Christiansen et al 2009; Axelstad et al

2011). In 2001 a group of scientists working for the national toxicology program defined low-dose exposure as 1) biological changes occurring at doses relevant to human exposure and 2) doses at lower concentration than used in standard testing protocols (Program and Agency 2001; Tse et al 2013; Christiansen et al 2014). A low dose exposure might lead to a different metabolic (toxic) response than seen as the normal s-shaped response curve, using doses up to maximum tolerated dose, as illustrated in figure 1. This phenomenon is called hormesis; where compounds show different effects at low concentrations compared to those at high concentrations (Program and Agency 2001). An example of hormesis is alcohol where it is believed that low daily doses may have beneficial effects, while high daily doses are unhealthy or even fatal.

In toxicological studies where rodents are exposed to low doses and up to maximum tolerable doses, toxic effects are often only detected at the higher doses. Below the NOAEL, no effects are seen. Herein, we applied a metabolomics approach to study EDCs in order to investigate whether changes to the metabolome could be detected at lower doses than those causing toxic effects. Therefore, metabolism and metabolites, systems biology, transcriptomics and metabolomics approaches will be briefly discussed below.

1.2 Metabolism and metabolites

Metabolism encompasses all the chemical and biological reactions necessary to maintain the living state of a cell or an organism. Compounds that potentially can cause an adverse effect can influence metabolism by e.g. disrupting regulatory pathways, blocking receptors or interfere with enzymatic reactions (Nielsen et al 2010b). Metabolism is also responsible for the modification and excretion of toxic compounds.

Metabolites are low molecular weight intermediates and products of metabolism, and the complete set of metabolites referred to the metabolome (Villas-Bôas et al 2007). It is believed that there are approximately 1000 metabolites in *E. coli* (Bajad et al 2006). In humans, the number of endogenous metabolites are estimated to be approximately 3000, present in concentrations ranging from picomolar to millimolar (Büscher et al 2009). The human metabolome data base hosted by Wishart *et al*. currently has more than 40.000 entries and include both endogenic and foreign metabolites (Wishart et al 2007; Wishart et al 2009; Wishart et al 2013).

In humans, food is absorbed by the intestine and transported through the blood to the target destination. The non-polar compounds are digested and converted into chylomicrons and transported through the lymphatic system to the liver from where it is distributed throughout the body (Berg et al 2006). The polar compounds such as free fatty acids are transported via serum albumin in the blood to reach their target organs. The liver is main site of metabolism. The liver enzymatically regulates the level of lipids in the blood

and removes foreign compounds by enzymatic modification followed by excretion in the urine. The intake of food and energy levels are regulated by enzymes. The concentrations of metabolites in the body are closely regulated e.g. by enzymatic reactions. The regulation of transcription is partly regulated by the concentration of metabolites, as depicted in Figure 2.

At the cellular level two types of metabolic reactions take place: anabolism ('building up') and catabolism ('breaking down'). Both reactions require countless chemical reactions. Anabolism requires energy for building molecules, whereas catabolism releases energy as molecules are broken down. The citric acid cycle – also known as the tricarboxylic acid (TCA) or Krebs cycle, is a catabolic reaction and is outlined below.

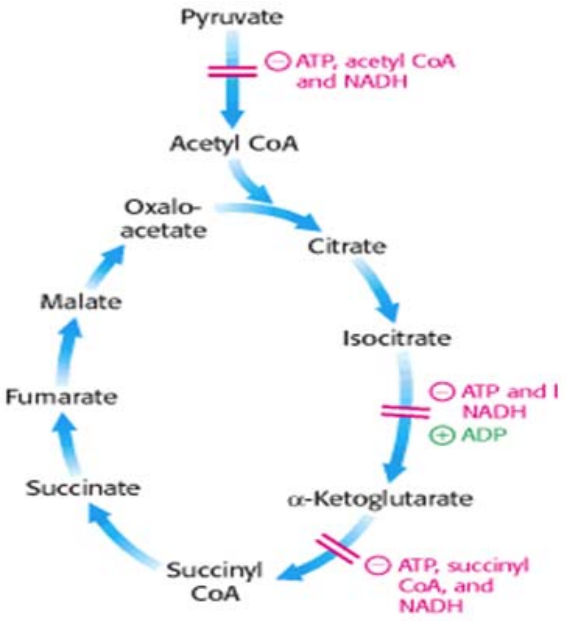

Figure 2 TCA cycle (Berg et al 2006)**. The blue arrows depict enzymes modifying the metabolites. As shown in the figure some enzymes are regulated by the concentrations of ATP, ADP, succinyl-CoA and NADH. If ADP concentration is high it might suggest a lowering of ATP and therefore an increase in the TCA cycle to produce more ATP. In the contrary, a high ATP concentration will inhibit multiple enzymes to reduce the ATP production**

The production of energy is tightly regulated by enzymes and the concentration of certain metabolites. A high ADP concentration will therefore induce energy production in the TCA cycle while an increased ATP concentration will inhibit multiple enzymes leading to a reduction in ATP production.

1.3 Systems biology

Systems biology aims to answer biological questions by applying a more holistic approach than what is done in more traditional reductionist approaches. It often combines traditional experimental technologies with powerful computer-based methodologies, which ultimately allows complex biological systems to be studied closer to its native state. Figure 3 depicts the central idea; measurements of core components give

input to understanding aspects of changes within an organism (Palsson 2009; Snyder and Gallagher 2009). To improve the understanding of how a given organism reacts to a xenobiotic, it is important to understand as much as possible of the complete system; genome, transcriptome, proteome and the metabolome.

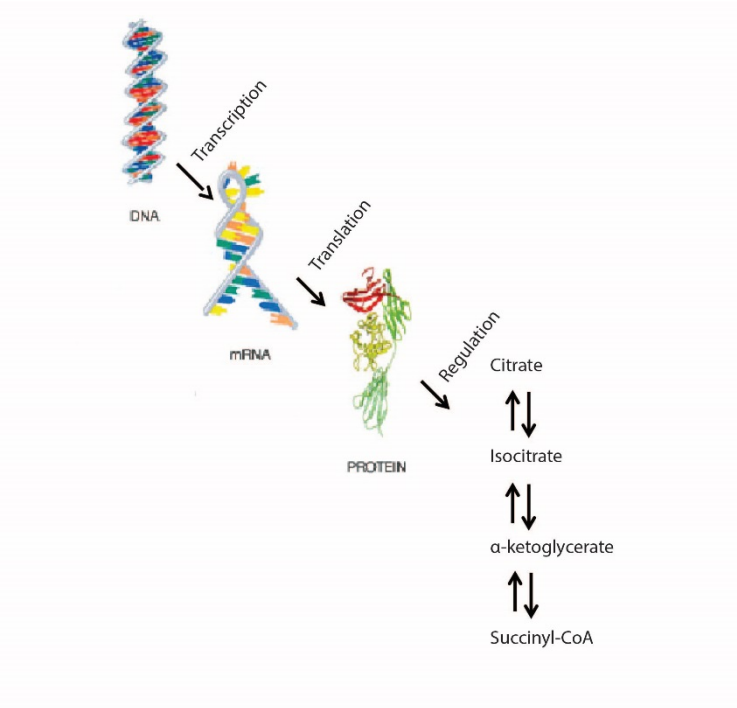

Figure 3 The classical central dogma used in systems biology. The connection between the DNA, proteins and metabolites (Palsson 2009; Snyder and Gallagher 2009)**.The genes are transcribed from DNA and controls the protein expression while the proteins are involved in the regulation of metabolites. Furthermore, the metabolites are feedback regulators for the gene and protein expression.**

The relationship between the 'omics' strategies is that DNA is transcribed into mRNA (transcriptomics), which is translated into proteins (proteomics), which is involved in the regulation of the metabolome (metabolomics). Concentrations of metabolites are involved in the control of enzymatic activity and verse visa. Furthermore, metabolites are involved in the regulation of transcription as well as translation of mRNA. Determination of all parts of the central dogma could reveal a full picture of how the body reacts at a given time point, though such measurements are close to impossible to conduct and furthermore if possible the cost would still be extremely high.

1.4 Metabolomics

The term metabonomics was introduced in 1999 by Jeremy Nicholson, Elaine Holm and John Lindon, who defined the term as "the quantitative measurement of time-related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modifications" (Nicholson et al 1999). A few years later, Oliver Fiehn introduced the term metabolomics and defined it as "the comprehensive and

quantitative analysis of all metabolites" (Fiehn 2001). An additional term, metabolic profiling, is also occasionally used as a name for the strategy of analyzing the metabolome. However, the term metabolomics is the predominant terminology. The objective of metabolomics is to determine the metabolome; the complete set of smaller metabolites (typically below 1-2 kDa) in a single organism (Oliver et al 1998; Goodacre et al 2004; Brown et al 2005). Therefore metabolomics must be seen more as an analytical strategy based on a comprehensive and quantitative analysis of all metabolites, than an analysis in its own right, similar to genomics (Lu et al 2008; Psychogios et al 2011; Robertson et al 2011). In reality, it is not possible to determine all sub-parts of the entire metabolome in one analytical set-up, hence a range of analytical strategies are used, including metabolite profiling and target analysis. While the term metabolomics is relatively new, the concept of metabolomics has been around for at least 2000 years as it was believed in ancient China that there was a correlation between health and body fluids and in the middle age "urine charts" were used to diagnose health conditions (Lindon and Nicholson 2008). The main indicators in these old "metabolic" studies was color, taste and smell of urine (Lindon and Nicholson 2008).

Mass spectrometry is the technique of choice for high throughput metabolomics (Feng et al 2008). Although, as mentioned above it is not yet possible to measure every single metabolite with a single analytical method (Shulaev 2006; Callahan et al 2009). A high throughput method for metabolome analysis can reveal information not obtained by the other "omics" approaches and could very easily be applied due to low cost (Gomase et al 2008). Furthermore, data obtained from metabolomics analyses are more phenotypic (Gomase et al 2008) compared to transcriptomics and proteomics. The combination of DNA sequencing, gene expression analysis, protein analysis and metabolite analysis could improve our understanding of complex systems, much more than the application of single method only. Analysing all parts simultaneously would possibly reveal a systemic change not observed using a single analytical method, ultimately increasing the likelihood of determining mode of action of toxic chemicals. However, many of these technologies are still very costly, at least if several are to be employed at the same time (Ramirez et al 2013). Also, current available technologies for metabolomics does not allow for an analysis of the complete metabolome.

The transcriptome and proteome have been analyzed in order to understand the effects of toxic compounds on gene and protein regulation (Wisselink et al 2010).

1.5 Use of metabolomics concepts in toxicology

In recent years, new techniques have been introduced to obtain more knowledge about the toxic effects of a compound. Concepts such as transcriptomics, proteomics and metabolomics are applied to clarify the mode of action of toxic chemicals (Monosson 2005). In toxicology, metabolomics approaches have been used over the last 15 years (Robertson et al 2011). The observation of metabolic changes can help understand effects caused by exposure to toxic compounds. At NOAEL, a metabolomics approach was shown to be more effective than conventional toxicology approaches in 15.4 % of cases, while in 70 % of cases the findings were similar (van Ravenzwaay et al 2014). Interestingly, it has been shown that changes induced by toxic compounds can be detected in the metabolome at NOAEL level e.g. drug induced changes in the gut microbiota and drug induced choline excretion into urine (Flores-Valverde et al 2010; Kozlowska-Tylingo et al 2010). The detection of perturbations in the metabolome following toxic exposure has been achieved on multiple different compartments of the human such as organs, blood and urine. However, of primary interest is blood and urine (Robosky et al 2002). Examples include the determination of blood parameters such as changes in cholesterol levels, low-density and high-density lipoprotein levels (Seidlová-Wuttke et al 2005; Hu et al 2010).

In toxicology, the focus of using metabolomics approaches to reveal toxic effects has shifted towards a more mechanistic-based approach, including determination of biomarkers for specific diseases (Robertson et al 2011). Analysis of the metabolome offers many advantages over conventional analytical methods for evaluating toxicity. “Since animals are sampled using a peripheral biofluid like urine, serial evaluation of the metabolic consequences of external stimuli such as the onset and regression of toxicity can be obtained from a single animal” (Robosky et al 2002). Metabolomics approaches have been used in the toxicology community to determine biomarkers of both renal (Sieber et al 2009) and hepatic toxicity (Antoine et al 2009; McBurney et al 2009). Furthermore, metabolomics approaches show great perspectives for analysis of low-dose exposure to toxic chemicals (Ramirez et al 2013). Hence, low-dose exposure to a compound such as BPA has been shown to induce changes of the polar fraction of compounds in the plasma (Zeng et al 2013) and to induce changes in the endoplasmic reticulum response in cellular cultures. Similarly, phthalates have also been shown to have endocrine disrupting effects at low-dose exposure, inducing anti-androgenic effects in male rats (Christiansen et al 2014).

Part II: Analytical challenges in metabolomics

2 Strategies in metabolomics

As discussed in section 1.4, metabolomics is a strategy used to determine the metabolome: all low molecular weight metabolites in a given sample at a specific time point (Fiehn et al 2007). This is an enormous task from an analytical perspective. The methodology is applied to mammals, plants, cell cultures and environmental systems.

The basic workflow of metabolomics employs a circular strategy (see Figure 4) where analytical chemistry is tightly integrated with biochemistry and informatics. The first step involves sample collection, quenching to stop all biochemical processes (if needed) and preparation of sample to match the subsequent analysis discussed further in section 2.2 and 2.3. The second step involves a chemical analysis to determine the metabolites in a sample by means of application of various analytical methodologies discussed in section 2.5. Subsequently, chemometrics is applied to classify samples, reveal differences between samples and to identify potentially relevant biomarkers, as discussed in section 2.6.

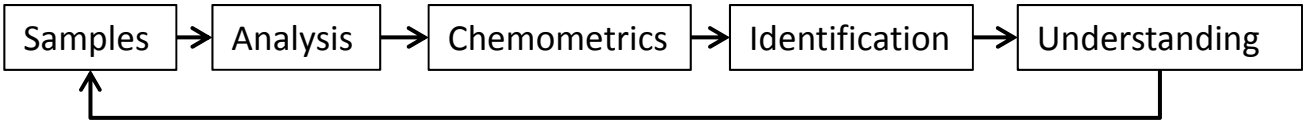

Figure 4 The paradigm in metabolomics. Samples representing the specific study are collected, quenched and prepared. Following the sample preparation they are analysed e.g. by LC-MS. Chemometrics tools are applied to locate metabolites expressed differently in the samples and these are attempted identified by e.g. accurate mass. From the identified metabolites and pattern of metabolites the goal is to achieve understanding of the metabolic effects.

2.1 The metabolomics platform

To create a platform for analysis of the metabolome, many aspects have to be considered. A general outline of the platform is illustrated in Figure 4. Urine or blood would be biological samples of choice, as they are quite accessible. Then decisions on how to prepare the samples must be considered. Finally, a choice of analytical instrumentation and data analysis method has to be made. For all of these steps there are pitfalls and problems, which will be discussed in the following sections.

2.2 Samples

Metabolomics has been applied to all types of body fluids, tissues and organs, including blood (serum and plasma), urine, feces, liver, skin and more (Souverain et al 2004; Psychogios et al 2011). Urine has been one of the most preferred samples due to the ease of collection, the stability of the samples, and because urine

reflects body excretion (the end-point of metabolism) (Zhang et al 2012b). Blood is not as easily accessible as urine, but nevertheless often used as it more accurately depicts the metabolic state of the body, e.g. by including temporal insight into the dynamic process of metabolism. On the analytical end, blood is also slightly more complex due to the large number of metabolites present, blood cells and proteins, as well as wide range in concentration and polarity of the metabolites (Dunn and Elis 2005; Psychogios et al 2011; García-Sevillano et al 2014). Tissues are more complicated, not least due to the fact that sampling normally involves an invasive procedure, e.g. through biopsies or recovery of cerebroventricular fluid (Tokushige et al 2013).

An additional complication when analyzing living organisms, or samples obtained from living organisms, is that the enzymatic systems need to be stopped to achieve a snapshot picture of the metabolome. The process employed to 'freeze' metabolism in time is referred to as quenching. For microorganisms it is very important to quench metabolism, as they are very easily stressed and the metabolitic concentration can change rapidly upon stress (Villas-Bôas et al 2005). For plasma samples, storage at -80^{O}C will arrest the enzymatic reactions, whereas protein precipitation will denature the enzymes.

2.3 Plasma analysis

Blood is the main transport medium of metabolites in the body. Hence, the plasma metabolome holds comprehensive information about the status of the various organs, cells and their regulation (Psychogios et al 2011). Plasma is obtained from blood samples by addition of anti-coagulants, followed by simple centrifugation and removing or decanting of the most buoyant (non-cellular) portion (Psychogios et al 2011). Plasma contains a significant chemical variety of metabolites from proteins and peptides, carbohydrates, lipids, nutrients, electrolytes, organic waste products from metabolism to a variety of small organic molecules (Psychogios et al 2011; Tulipani et al 2013). This chemical diversity of metabolites is by far the largest challenge in plasma analyses (Souverain et al 2004). Furthermore, a substantial number of metabolites in the body have the same exact elemental composition. Therefore, it is difficult to segregate and identify these metabolites by for instance mass spectrometry.

Many different analytical strategies have been used (Psychogios et al 2011) to gain access to the plasma metabolome, typically by removing some of its components in order to achieve good chromatographic separation and sensitive mass spectrometric detection. A simple cleanup method involves precipitation of proteins with an organic solvent e.g. methanol, perchloric acid and acetonitrile (Souverain et al 2004). It has been suggested that three volumes of ice cold acetonitrile per plasma volume is the best solvent for protein precipitation (Gage and Stopher 1998; Souverain et al 2004; Michopoulos et al 2010; Tulipani et al 2013). The combination of protein precipitation with liquid/liquid extraction has been done with success: Bird *et*

al. used hexane to extract lipids after protein precipitation, while Ravenzwaay *et al.* used a mixture of ethanol/dichloromethane with water to separate the polar and non-polar fraction (Bird et al 2011; van Ravenzwaay et al 2014). As liquid/liquid extractions have been used for decades, a variety of different solvent combinations have been proposed (Takatera et al 2006; Sandra et al 2010; Ferreiro-Vera 2012).

An alternative to liquid/liquid extraction is solid phase extraction (SPE), using a solid phase to retain or remove the compound of interest. As suggested by Tulipani *et al.* SPE can be used to remove phospholipids after protein precipitation to increase the information of the remaining metabolites (Tulipani et al 2013). This effect is due to the fact that phospholipids are extremely easy to ionize in electrospray mass spectrometry, and will therefore give a significant bias towards phospholipids (matrix like effect). Removing these will most likely enhance the determination of the remaining metabolites (Ferreiro-Vera 2012; Tulipani et al 2013). One of the challenges in analyzing more complex samples such as plasma, is the wide range in the concentration the metabolites which goes from picomolar to millimolar (Psychogios et al 2011). For the purpose of identifying all metabolites in plasma, purification or cleanup of the plasma samples might be necessary. As described, a simple approach to remove proteins, or a more complex approach using SPE or liquid/liquid extraction, can be used. However, each will have advantages and disadvantages. For SPE purification, it is possible to selectively isolate one specific class of compounds, though with the risk of losing some of the analytes of interest. The simple sample preparation will have the advantages that most of the compounds of interest will be preserved and that steps required for the more complicated purification steps might change the compound concentration of the sample. The disadvantage of the simple sample preparation is that some of the metabolites might be lost in the analysis of the sample due to e.g. ion suppression. A purification step of a complex sample can be a good choice to detect possible changes in the organism though some information might be lost due to the purification process.

2.4 Targeted versus non-targeted metabolomics

Broadly speaking, two different approaches are used when evaluation toxicity by metabolomics: i) a targeted approach that aims to determine a preselected set of metabolites and ii) a non-targeted approach that aims to determine a broad range of metabolites. In both cases it is highly desirable to do a relative quantification of the metabolites. Since targeted metabolomics focuses on a selected class of metabolites, it allows for a more elaborate sample preparation, including up concentration using e.g. SPE, liquid-liquid extraction or derivatization to achieve a high sensitivity approaching 1 pM for some metabolites (Wishart 2010). The non-targeted approach is normally based on a screening or fingerprinting strategy where all detectable metabolites are considered. This could involve a crude extraction procedure followed by injecting the extract directly in a mass spectrometer or rough LC-MS techniques (Robertson et al 2011).

While the non-targeted metabolomics approach may reveal more information from each sample, the detection limit is far higher than that of the optimized target approach (100-200 nM according to Wishart 2010). In both cases, it is critical to note that the metabolome data obtained depends on the applied analytical procedure unless a validated quantitative target approach is employed. The non-targeted metabolomics approach presents a complex fingerprint of the sample that can be efficiently used to classify samples. However, in the non-targeted approach it can be difficult to classify an identified metabolic profile (García-Sevillano et al 2014), as the differences usually are small compared to major component (e.g. dominant primary metabolites). In the targeted approach, selected sets of metabolites are determined, with increased sensitivity and also allow for quantification. However, the most pronounced disadvantage for targeted metabolomics is that only known metabolites are detectable (Ebbels and Cavill 2009; Robertson et al 2011).

2.5 Instrumental setup

Several different analytical methodologies have been used to analyze metabolomes, including NMR, LC-MS, GC-MS and CE-MS. NMR has been applied in metabolomics for measuring polar compounds such as amino acids and organic acids. Each of the three methods, NMR and LC-MS or GC-MS, have inherit advantages and disadvantages and neither method can cover analysis of the full metabolome (Brown et al 2005; Psychogios et al 2011). Therefore, data obtained by metabolomics is dependent on methodology and data cannot be directly compared across methodologies.

The primary use of NMR has been to analyze proton spectra, although methods analyzing C^{13} at natural abundance or enriched has also been applied (Robertson et al 2011). NMR is a strong analytical tool as it can quantify many metabolites during a single analytical run. NMR has some unique advantages such as being non-destructive and non-biased, as well as offering easy quantification (Wishart 2008). Moreover, it provides molecular structures, thus giving certainty in the identification of metabolites as compared to mass-based identification. One drawback is the fitting of overlapping peaks, though recent research have developed peak fitting for library searches (Wishart 2008). Furthermore, NMR yields relatively low sensitivity and large sample sizes are needed, typically 500 µl for plasma (Wishart 2008; Lu et al 2008).

GC-MS has been used in metabolomics to analyze multiple compounds, from sugars to lipids. GC-MS relies on electro impact ionization that breaks the analyte into many fragments, which can be used to identify the metabolites. One drawback is the need to render all analytes more volatile, generally through derivatization chemistry, introducing an additional analytical variable (Gomase et al 2008). Non-volatile compounds which do not generate derivatives, that are large and/or thermo-labile, will not be detectable in gas chromatography (Zhang et al 2012a). Derivatization of the compounds, however, has the advantage

that it can assist in structure identification. Furthermore, GC-MS will yield the same spectra using different instruments, such that databases can be created to conduct fast and accurate identification. GC-MS has been used since the early 1970's and ever since the development of mass spectrometry the use of GC has proven to be a strong tool for analysis of a wide range of compounds (Fiehn 2008). Hence, GC-MS is a widely used and powerful method, however only volatile metabolites can be analyzed without derivatization (Gomase et al 2008; Zhang et al 2012a)

HPLC-MS has been used to study metabolites such as organic acids, sugars, lipids and phospholipids among others. HPLC-MS is a strong tool that can analyze a wide polarity range. One major drawback is the identification of unknown metabolites. Compared to GC-MS where similar fragmentation patterns are revealed, even on different instruments, HPLC-MS does not necessarily show same MS pattern on different instruments. Therefore, it can be difficult to identify the metabolite doing non-targeted metabolomics. Furthermore, ionization using electrospray ionization (ESI) will induce insource fragments along with adducts which can impend the identification. On the other hand, such adducts and fragments can assist in identifying pseudo-molecular ions

High performance liquid chromatography (HPLC) is widely used, as it allows for a broad-range screening of analytes. The polar compounds (e.g. amino acids analyzed by HPLC) will normally be separated using normal phase chromatography. The normal phase has a polar stationary phase retaining the polar compounds. A method for analyzing very polar compounds such as adenosine-triphosphate is available: hydrophilic interaction chromatography (HILIC), which is a part of normal phase chromatography. In HILIC the stationary phase is surrounded by a water layer which assists in the separation of polar compounds (Alpert 1990; Ta et al 1994). For analysis of more non-polar compounds such as lipids, reverse-phase chromatography is used. The reverse phase has a non-polar stationary phase retaining non-polar compounds. The columns used for reverse phase chromatography is primarily C18 and C8 columns.

RP-HPLC is an analytical technique with high reproducibility and sharp chromatographic peaks. RP-HPLC combined with mass spectrometry has been used to analyze a wide range of compounds e.g. phospholipids, neutral lipids and many more. Ikeda *et al* analyzed triacylglycerols to increase understanding of MSMS patterns of these. Others have used RP-HPLC to investigate changes of the lipodome in diseases and upon exposure to toxic chemicals. Ang *et al* analyzed day to day variation of phospholipids and free fatty acids of human plasma (Ang et al 2012) while Garcia-Sevilliano *et al* have shown changes in the lipodome upon toxic exposure of inorganic arsenic (García-Sevillano et al 2014). RP has a wide application in metabolomics and can analyze a wide range of metabolites. One of the disadvantages is its poor separation of polar compounds. However, this can be solved by HILIC which can separate and detect

extremely polar compounds (Bajad et al 2006; Callahan et al 2009). Using a HILIC system, one faces problems with peak shape, ion suppression from the high ion contents in the solvent and poor reproducibility.

In recent years two different kinds of analysis have been used, the 2 dimensional analytical approaches GC-GC and LC-LC. GC-GC is a powerful technique which has gained increasing attention over the last two decades, and can provide greatly increased separation capacity, chemical selectivity and sensitivity for complex sample analysis, bringing more accurate information about compound retention times and mass spectra (Hu et al 2010). LC-LC is a good separation technique for polar compounds as described by Klavins *et al* where a combination of an anionic exchange and a porous graphic column to separate compounds such as sugar phosphates and small organic compounds (Klavins et al 2014). The drawback of a LC-LC method is a significantly longer analytical runtime.

Today, MS is probably one of the most important analytical techniques within biotechnology, analyses ranging from small volatile compounds, complex natural products and proteins to intact viruses (Villas-Bôas et al 2007). High resolution instruments can now possibly detect pM with a resolution of 100.000 and a mass accuracy when internally calibrated at below 1-2 ppm (Lu et al 2008). The use of high resolution mass spectrometry increases the accuracy of the mass spectra, reducing the amounts of suggested metabolites when conducting database searches. The high resolution mass spectrometer is useful for analysis of complex samples due to resolution, accuracy and sensitivity (Moco et al 2007). Using a qTOF instrument, the accurate mass along with MS^2 can help identify compounds but also possible impurities in the chromatography (Wolff et al 2001). For the analysis of well characterized samples, a simple mass spectrometer (e.g. a single quadrupole) might be more robust and thus yield more reliable results for each sample. For identification of new compounds, this instrument will not be useful while a high resolution instrument can narrow down the amount of possible compounds, thereby increase the likelihood of identification. The use of high resolution mass spectrometry can be a powerful tool for identification of compounds even from a complex matrix.

2.6 Data processing and statistical methods

Metabolomics, especially non-targeted, creates a large amount of data. Chromatographic data has to be analyzed and for statistical analyses the data has to be sorted into a matrix containing retention times, m/z values and intensities. To create a matrix, multiple tools are needed, such as time alignment of the chromatography, scaling of the data and reduction of noise (Idborg et al 2005). Time alignment ensures that a possible shift in analysis time over the full analytical run will be aligned so that the compounds can be analyzed in the same “bucket”. In turn, the bucket is created to form the matrix. Scaling is performed to

homogenize the data as much as possible. Lastly, since a large proportion of the data can be noise, a reduction of the noise can reduce the time for identifying differences in the metabolome. To identify these differences, statistics is a useful tool. Particularly for non-targeted metabolomics, statistical methods such as principal component analysis (PCA) and partial least square regression (PLS) are used (Ebbels and Cavill 2009; Robertson et al 2011).

Both unsupervised and supervised statistical tests are applied in metabolomics. The latter includes analysis of variance (AVONA) and PLS, while the former includes PCA and hierarchical clustering (Shulaev 2006). The unsupervised statistical tests are used to detect patterns in data with no predefined control and exposed groups. Furthermore, they can be useful to detect outliers which can then be removed from the dataset. In a supervised statistical test, the statistical model has predefined knowledge about control group and exposed group. In the present work ANOVA, PLS and PCA have been applied.

T-test

The Student's *t*-test aims to identify differences between two groups, and is only applicable if the data are normally distributed. Data that are not normally distributed can be analyzed with nonparametric tests such as Mann-Whitney (Comparisons and Dice 2002). In order to analyze more than two groups, analysis of variance (ANOVA) can be performed. ANOVA can be used to analyze groups of the same substance and correlate these results to that of a control group e.g. groups of animals dosed with the same substance in increasing amounts.

PCA and PLS

Models are often applied to metabolomics data to further illustrate the differences to separate metabolomes. One such model is a PCA (Ebbels and Cavill 2009). PCA is a projection technique which is designed to extract, display and rank the variance in a dataset. The purpose is to reduce the dimensionality while at the same time retain the information in the dataset. The PCA filters out the most significant variables in the original matrix so instead of looking at a large number of variables the PCA allows identification of the most influential variables. Another model used for interpretation of changes in metabolism is the PLS regression (Shulaev 2006; Bartel et al 2013). PLS analysis also uses a collection of analyses which is translated into a matrix. The number of X variables corresponds to the number of column in the X table. However, a second table with sample information is also taken into account. Hence, PLS is referred to as a statistical supervised method. Unlike PCA, which detects the direction of maximum variation, PLS attempts to identify the best correlation between X and Y tables using linear combinations of variables in x and y table (Ebbels and Cavill 2009). When using PCA it is important to check if the model can

separate groups. If the PCA model cannot distinguish between the two groups it is unwise to use more sophisticated models, as these might just over-fit the model and introduce possible errors into the model (Wishart 2010).

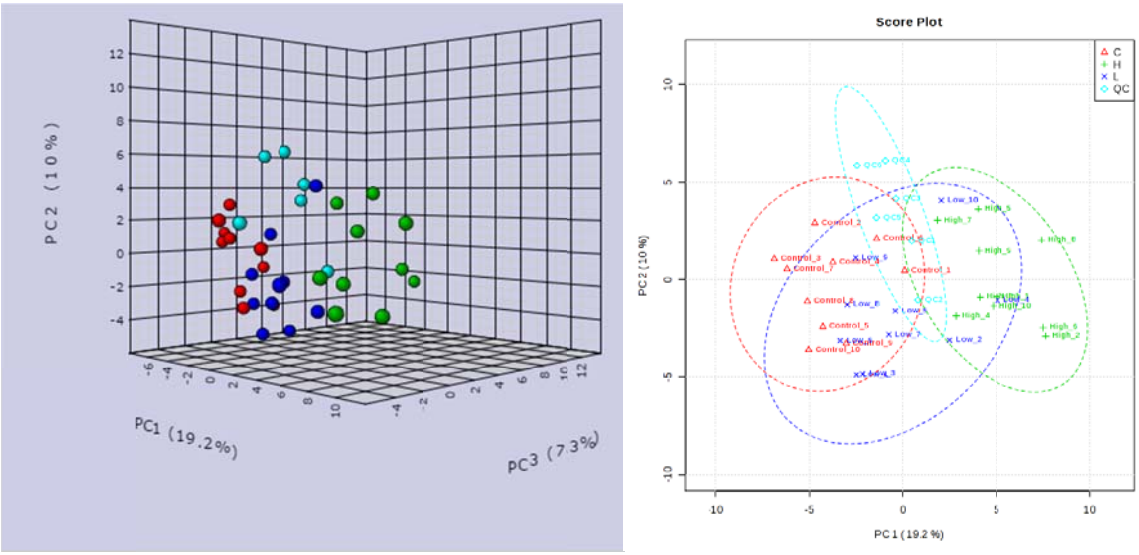

Figure 5 3 D and 2 D score plots of male BPA animals. The third dimension illustrates how the groups are related to each other. Both the plots display differences between the groups, though the third dimension really separates the high dose animals from the low dose and control.

The application of statistical analysis is an important step in metabolomics. Especially in non-targeted metabolomics, the identification of changed metabolites is based on statistical models such as PCA or PLS. In the present study PCA, ANOVA, t-test and PLS have been applied (Shulaev 2006; Ebbels and Cavill 2009; Robertson et al 2011; Bartel et al 2013). Using statistical approaches for non-targeted metabolomics helps with the identification and visualization of the compounds of interest and thereby to identify "the needle in the haystack" (Ebbels and Cavill 2009).

2.7 Challenges with interpreting metabolomics data

Metabolomics has been used to evaluate a range of different diseases and toxicities. In the present study metabolomics has been applied to detect changes in the metabolome upon low dose toxic exposure. To conduct analysis of animals with respect to both toxicity and disease, it is important to know how the metabolome is affected by factors such as age, gender, strain variations and so forth. As metabolomics typically looks at a snapshot of the metabolome, it is important not to induce unwanted variation between samples reflecting the above issues as well as technical variation. For instance, it has been shown that control animals from two different rooms at the same facility can have different metabolic profiles (Robertson et al 2011). This highlights the need to be very careful when conducting metabolomics analyses.

For sample preparation certain factors have to be controlled. Temperature, duration, freezing, thawing, order of analysis are some of the factors that can affect the results. Thawing samples at room temperature compared to thawing on ice has been shown to change the metabolite composition (Breier et al 2014). However, for many lipid classes in the plasma metabolome, the stability is high even at 4 degrees for 6 hours (Breier et al 2014). For analysis of primary metabolites in microorganisms it has been shown that quenching is required to ensure that the metabolite pool stays unchanged (Villas-Bôas et al 2005). Another factor which has great impact is gender; a study of pH effects on muscle showed a difference in response between male and female muscles (Ellis et al 2014). Furthermore, Jeremek e*t al.* have shown that men and women respond differently to alcohol consumption (Jaremek et al 2013). Using mass spectrometry allows for randomization of the samples, as the mass spectrometer will have a reduced sensitivity over time due to reduction of signal in the detector of the mass spectrometer. It has been shown that the instrument run-time in an experiment with many samples can be one of the critical factors affecting variance (Burton et al 2008). To ensure that no drifts or malfunction are occurring during the analytical run, a number of quality control samples reflecting the number of samples are included in the analyses. At the end of the analytical run the quality control sample must cluster closely in the score plot (Burton et al 2008).

Many studies aim to identify possible biomarkers of diseases, for early detection of illness in humans. For a range of diseases and exposure to toxic chemicals, biomarkers have been identified (Singh and Li 2011; Austdal et al 2014). A biomarker reflects changes in a normal biological process and can assist in the diagnostics of disease (Larson et al 2013). Studies have been conducted where an analysis of the metabolites is performed in order to understand how diseases affect the metabolome (Chen et al 2014). Biomarkers have been identified for diseases such as cancer, Alzheimer's disease, diseases associated with pregnancy and Parkinson's disease (Michell et al 2008; Yonezawa et al 2013; Kobayashi et al 2013; Austdal et al 2014). Metabolic changes have been analyzed in the hope of identifying biomarkers of diseases, to determine early stages of the diseases (Zhang et al 2012b; Jung et al 2013). A number of biomarkers have been identified e.g. blood glucose levels as biomarkers for the risk of acquiring diabetes, serum creatine for kidney failure and prostate specific antigen for prostate cancer (Xia et al 2013a). Metabolomics as an approach for identification of biomarkers is widely used. But although many biomarkers for diseases and toxic effects have now been suggested, statistical evidence are often lacking (Leichtle et al 2013).

3 Part III: Research objectives and biological questions being asked

The aims of this study were to achieve new insights into the action of toxic compounds on biological systems. A metabolomics approach was applied. The primary aim was to develop a sample preparation procedure and LC-MS analyses to detect as many metabolites as possible in the plasma metabolome. Further to use this procedure for the analyses of plasma from rodents exposed to combinations of endocrine disrupting chemicals for identification of metabolites changed in concentrations which could serve as biomarkers of toxic effects. Also, to investigate whether changes in concentrations of these biomarkers could be observed using metabolomics at lower exposures not resulting in toxic effects. Specifically to:

- Development of an analytical method for determining changes in the metabolome due to low-dose exposure to toxic chemicals
- To illustrate the efficiency of the analytical method, and if the analytical platform can be used to identify and evaluate low- and high-dose exposure effects to toxic chemicals
- Can metabolomics be used to extrapolate data from low- to high-dose exposure?
- Is it possible to identify mechanism of action for low dose exposure to a toxic chemical?

4 Part IV: Material and methods

4.1 Sample collection

The Animal study was carried out at the DTU National Food Institute (Mørkhøj, Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given: 2012-15-2934-00089 C4. The experiments were supervised by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

4.1.1 PFNA experiment

Male Wistar Hannover Galas rats, six weeks of age with pathogen-free health status, were obtained from Taconic M&B (Lille Skensved, Denmark), and allowed to acclimatize for one week. The animals were housed two per cage (Macrolon, Buguggiate, Italy) with light on from 7 am to 7 pm. Room temperature and relative humidity were 22±1 °C and 55±5%, respectively. Rats were given ad libitum access to acidified tap water and standard diet (prod. no. 1324 Altromin, Brogården, Gentofte, Denmark). The animals were administered test substances once a day by gavage for 14 days with corn oil (VWR - Bie & Berntsen, Herlev, Denmark) as vehicle. The dosing volume was 1 mL/100 g of body weight (bw). At the end of the experiments, animals were anaesthetized in CO_2/O_2 and decapitated. Neck blood was collected in heparinized tubes, and plasma was isolated by centrifugation at 1,000 x *g*, 4 °C for 10 min. Plasma was stored at -80 °C. Around 1 ml of plasma from each of the 82 animals were obtained.

4.2.1 BPA experiment

A total of 110 time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at gestation day (GD) 3 of pregnancy.

The animals were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden). The study was performed using three blocks (separated by 1 week), and all dose groups were equally represented in the blocks, i.e. the 22 time-mated rats per dose group were allocated among blocks. The dams were distributed into five dose groups (0, 0.025, 0.250, 5 or 50 mg/kg bw per day respectively). Details on study design are described in Christiansen et al. (2014).

At the end of the experiments, animals were anaesthetized in CO_2/O_2 and decapitated. Neck blood was collected in heparinized tubes, and plasma was isolated by centrifugation at 1,000 x *g*, 4 °C for 10 min. Plasma was stored at -80 °C. Around 0.1 ml of plasma obtained from each of the 90 animals.

4.1.3 Selenium experiment

Urine samples were obtained from a previously reported Se bio-distribution study (Loeschner et al 2014)**.** Briefly, four-week-old, specific pathogen-free (SPF) female Wistar rats were obtained from Taconic M&B (Lille Skensved, Denmark). The rats were allowed to acclimatize for one week. The rats were housed in pairs with a 12:12-h light/dark cycle with the lights on from 7 a.m. to 7 p.m. The room temperature was 22 ± 1 ^{o}C, and the relative humidity was 55 % ± 5 %. The rats were given ad libitum access to a standard diet (Prod. no.1324, Altromin International, Lage, Germany) and citric acid acidified tap water.

4.2 Sample preparation

The method was based on SPE followed by chemical separation. As illustrated in figure 6 the plasma was thawed on ice, followed by protein precipitation with three volumes acetonitrile containing 1 % formic acid. The sample was centrifuged at 10000 g to remove proteins and supernatant added to a hybrid SPE phospholipid column (30 mg Hybrid SPE Phospholipid column Supelco, Sigma–Aldrich, St. Louis, MO). The SPE column was first eluted by the addition of 300 µl of 1% formic acid in acetonitrile, and the eluate was collected in a vial and evaporated to dryness at 50 °C using a gentle stream of nitrogen. The dry residue was then extracted by adding 200 µl of heptane. The heptane fraction was then transferred to a clean vial. The residue was then extracted by adding 200 µl of methanol (polar fraction). The heptane fraction was evaporated at 50 °C using a gentle stream of nitrogen. The dry residues of the heptane fraction was dissolved in 200 µl of 50:50 (acetonitrile:isopropanol) (lipid fraction).

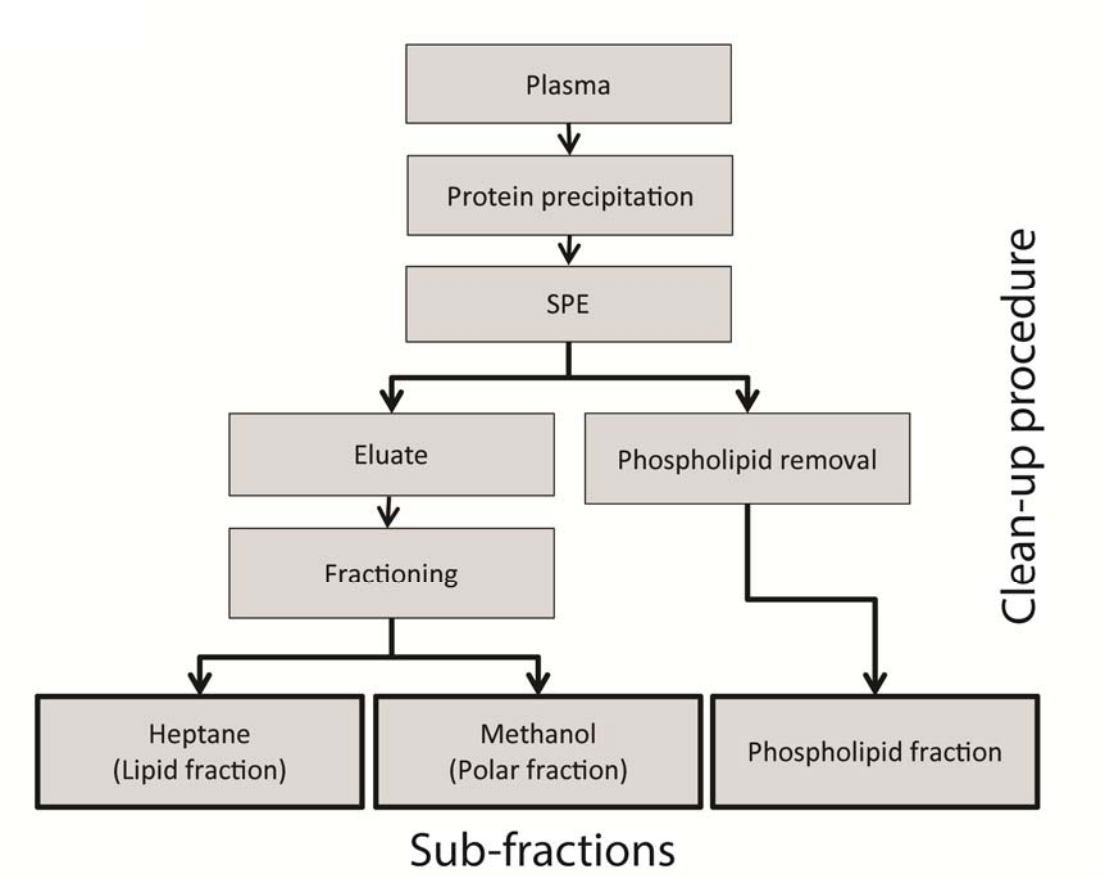

Figure 6 Flow diagram of the SPE method. Proteins were precipitated by adding 300 µl acetonitrile with 1% formic acid followed by SPE clean-up of the supernatant in three sub-fractions.

The hybrid SPE column was eluted by 300 µl of 10% ammonium hydroxide in methanol, in order to elute the phospholipids. The phospholipid fraction was diluted with 200 µl Milli-Q water and 500 µl of methanol.

4.3 Sample analysis

4.3.1 High pressure liquid chromatography

The analysis was conducted on a Dionex Ultimate 3000 RS (Thermo Scientific, CA) combined with a Maxis qTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The solvents used for the HPLC analyses were A: 0.1 % formic acid + 5 mM NH_4OH in water and B: 0.1 % formic acid in acetonitrile. The flow was 0.3 ml/min and two different solvent gradient systems were used, as described below. The HPLC column was an Ascentis Express C8 (100x2.11 mm, 2.7 µm) column (Supleco, St. Louis, MO).

Table 1 The two gradient systems used in the analysis (Paper I-IV). The hydrophilic programming was used for the polar fraction while the hydrophobic programming was used for the phospholipid and lipid fraction.

Hydrophilic	Hydrophobic
0 min; 0 % B	0 min; 70 % B
3 min; 5 % B	3 min; 75 % B
10 min; 100 % B	8 min; 100 % B
12 min; 100 % B	10 min; 100 % B
12.1 min; 0 % B	10.1 min; 0 % B
14 min; 0 % B	12 min; 0 % B
Injection volume 3 µl	Injection volume 1 µl
Temperature 40^{O}C	Temperature 50^{O}C

4.3.2 Mass spectrometry

The settings for the mass spectrometer can be found in the appendix (**appendix II**). The LC-MS data were evaluated using Bruker Daltonics Data analysis 4.0 and/or 4.1. For the multivariate data analysis of mass spectra, Profile Analysis 2.1 combined with metaboanalyst.ca was used. The data matrix was created by the "Find Molecular Feature" using a mass range from 50 to 1100 Da, combined with a time alignment function and a retention time window from 30-720s and 30-840s, for the hydrophilic and hydrophobic gradient system, respectively.

For the quantification of PFNA in the plasma samples, Quantanalysis 2.0 (Bruker Daltonics) was used. To quantify the PFNA concentrations, a standard curve was constructed between 10 ng/ml and 700 µg/ml. The high concentration PFNA samples were diluted to fit in the linear range (**Paper II)**. For the analyses of plasma samples from animals dosed with BPA, a standard curve between 0.01 µg/ml and 300 µg/ml was created; however BPA was not detected in the samples (**Paper IV**).

MSMS analysis was performed using the "auto MSMS" function in the data analysis software (Bruker Daltonics, Bremen, Germany). From each group of animals were two samples selected and MSMS performed. The auto MSMS function was set to ramping fragmentation energy from 10-30 eV in the mass range of 100-1000 Da e.g. a mass of 100 Da would have 10 eV while a mass of 1000 Da would have 30 eV fragmentation energy. The auto MSMS creates MSMS patterns for every second scan on the top 3 intensity peaks.

4.3.3 NMR analysis

An NMR analysis was conducted on plasma from the PFNA exposed animals (**Paper III**). Centrifugal Filter Units (Merck, Millipore, Darmstadt, Germany) was washed 5 times with 500 µl water to remove excess glycerol from the production. Five hundred µl of plasma was filtered by centrifugation at 10.000 rpm at 4^{O} C for 35 min. Two hundred eighty-five µl of the filtrate was transferred to an Eppendorph vial along with 35 µl D_2O and 30 µl 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) (internal standard). The sample was transferred to a Shigemi tube and analyzed by H-NMR. H-NMR settings can be found in (**appendix II**).

4.3.4 Gene analysis

Total RNA from six rat livers each from vehicle control, Low PFNA + Mix, and Mid PFNA ± Mix groups were separately converted into labeled cRNA and applied to the One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) version 6.5 (Agilent Technologies, Santa Clara, CA). Labeled cRNA from each rat was hybridized to Agilent Whole Rat Genome Oligo Microarrays (G4122F) for 17 h at 65 ^{O}C. The hybridized microarrays were scanned using an Agilent DNA Microarray Scanner and evaluated using the Feature Extraction software version 10.7.3.1 according to protocol GE1_107_Sep09 (Agilent Technologies) to generate feature extraction files for further analysis. Reads were quality controlled by the software prior to release of the data. Arrays that did not pass quality control were removed from the dataset. Based on the quality control reports, two of the six microarrays from the Mid PFNA + Mix group were excluded from further analysis. The remaining arrays, six from each of control, Low PFNA + Mix, and Mid PFNA and the remaining four from Mid PFNA + Mix were found to be of high quality. Extracted data were analyzed using the limma software package (Smyth 2004; Smyth 2005) in R (R Core Team 2012). Data were background corrected using the 'normexp' method (Ritchie et al 2007) and normalized between arrays using quantile normalization (Smyth and Speed 2003) prior to statistical analyses. Within-array replicate probes were replaced with the average expression level. To identify treatment-specific gene effects, we fitted a linear model for each gene and applied empirical Bayes statistics (Smyth 2004) for each relevant two-group comparison. The false discovery rate (FDR) was controlled using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). Reported p-values for the significantly differentially expressed genes from the transcriptomics analysis were all adjusted, and p-values below 0.05 were considered statistically significant.

4.4 Identification processes

Identification of metabolites differing between dosed and control animals is an important aim in the metabolomics investigation. To understand the change in non-targeted metabolomics, identification of the changed metabolite is required. For the identification of unknown metabolites, databases which can assist

in the identification are available. The metabolomics community has focus on projects aiming at describing metabolites, e.g. projects such as The Serum Human Metabolome Project (HUSERMET) (Zelena et al 2009) and the Metabolomics Standard Initiative (MSI) (Fiehn et al 2007). The collective work of these projects has led to an information collection which is easily accessible from databases and other web-based programs, such as human metabolome database (HMDB.ca) (Wishart et al 2007; Wishart et al 2009; Wishart et al 2013), Kyoto Encyclopedia of Genes and Genomes (KEGG.jp) (Kanehisa and Goto 2000), MassBank (MassBank.jp), METLIN (metlin.scripts.edu) (Trauger et al 2005; Smith et al 2005) and Lipidmaps (Lipidmaps.org) (Sud et al 2007; Fahy et al 2009). These projects and databases can help to identify metabolites from their accurate mass. Projects combining metabolomics data with transcriptomics data have been conducted, e.g. InMex (InMex.ca) (Xia et al 2013b). Such tools can, along with the knowledge of pathways and systemic changes, help to identify signs of sickness or health risk assessment (Xia et al 2013b).

4.5 Metabolite identification

Metabolites found to be significantly different between exposed and control animals were searched for in databases such as HMDB (Xia et al 2009; Xia et al 2012; Wishart et al 2013), Lipidmaps (Fahy et al 2009), Metlin (Trauger et al 2005) and MassBank. The identification of metabolites (**Paper I-IV**) was corroborated by MSMS analysis using an auto MSMS approach. The MSMS spectra were acquired using the Maxis qTOF instrument with a linear collision energy gradient from 10 to 30 eV in the mass range of 100-1000 Da. The auto MSMS settings used created MSMS spectra for the three highest intensities in the MS spectrum. This is useful for 95 % of the compounds; however, low intensity peaks could be difficult to identify when using this approach. To identify the last 5 % a list of the compounds of interest would have to be created for a specific MSMS analysis of these compounds. For a sub-fraction of the significant metabolites MSMS patterns and retention times were compared with similar data obtained from analysis of a purchased standard.

To identify the unknown metabolites the following procedure was used:

1) A mass was extracted from the statistical analysis with four decimal accuracy
2) The mass was searched for using HMDB, Metlin, MassBank and/or KEGG (±20mDa)
3) Possible candidates were suggested and if possible, a MSMS pattern was obtained from databases (lipidmaps or HMDB)
4) The identified MSMS pattern was compared with the MSMS pattern created using the qTOF instrument – if a match compound was marked as identified, see Figure 7

5) For some compounds, e.g. MG(18:0) and MG(16:0), a standard were purchased to verify the identity of the compound
6) If a compound could not be identified, a m/z value was assigned

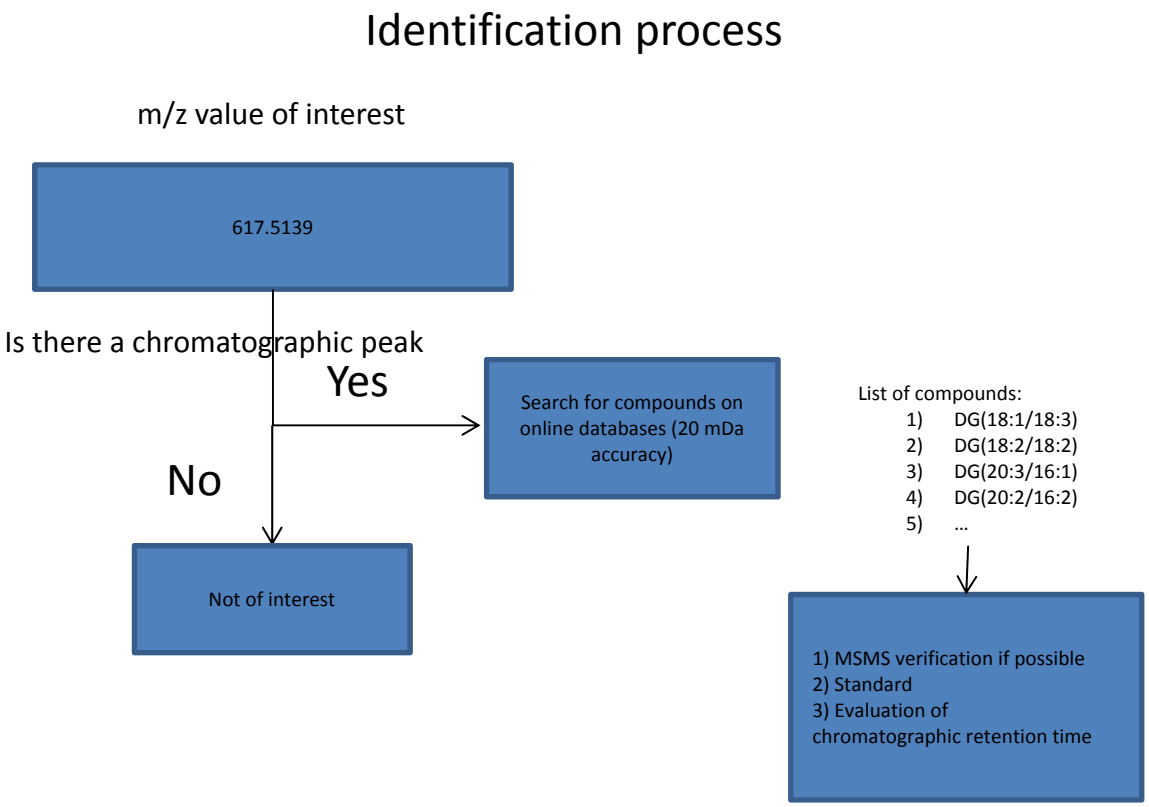

Figure 7 Identification process. A mass of interest is identified. The chromatographic peak of the specific mass is evaluated. The mass is searched for in online databases by accurate mass with an accuracy of 2 or 20 mDa. For this specific example 29 suggested metabolites are found using either 2 or 20 mDa. A list of suggested compounds is created and reduced by MSMS, standard and retention time of the compound. A compound is identified if a MSMS pattern can be correlated or in best case if a standard is purchased.

Two examples of identification are shown in Figure 8. The top (a) spectrum shows the MSMS spectrum of an ion with a mass of 634.5402. The MSMS spectrum shows in addition to the parent ion the pseudo molecular ion (M+H)+ (m/z 617.5139) along with a loss of water (m/z 599.5033), furthermore two fragments of 337.2735 and 269.2369 are found. HMDB suggest DG(18:2/18:2) as a possible $M+NH_4$ ion; which could be correlated to the neutral side chain loss (m/z 337.2735) and loss of the acyl side chain (m/z 263.2369) along with loss of water. Comparing this MSMS pattern with the *in silico* MSMS pattern of DG (18:2/18:2) from Lipidmaps shows that all the fragments fit and that the mass deviation is less than 0.6 ppm for all of the fragments. The compound was identified to be DG(18:2/18:2) as it only has one neutral loss of the side chain, there are multiple other compounds with the same mass e.g. DG(18:1/18:3) or

DG(16:2/20:2) though as there is only one fragment around 337.27 it has to be a loss of a 18:2 sidechain. If the compound were DG(18:1/18:3) the MSMS pattern would have a mass of 335.27 and 339.28 comparing to loss of 18:3 and 18:1, respectively.

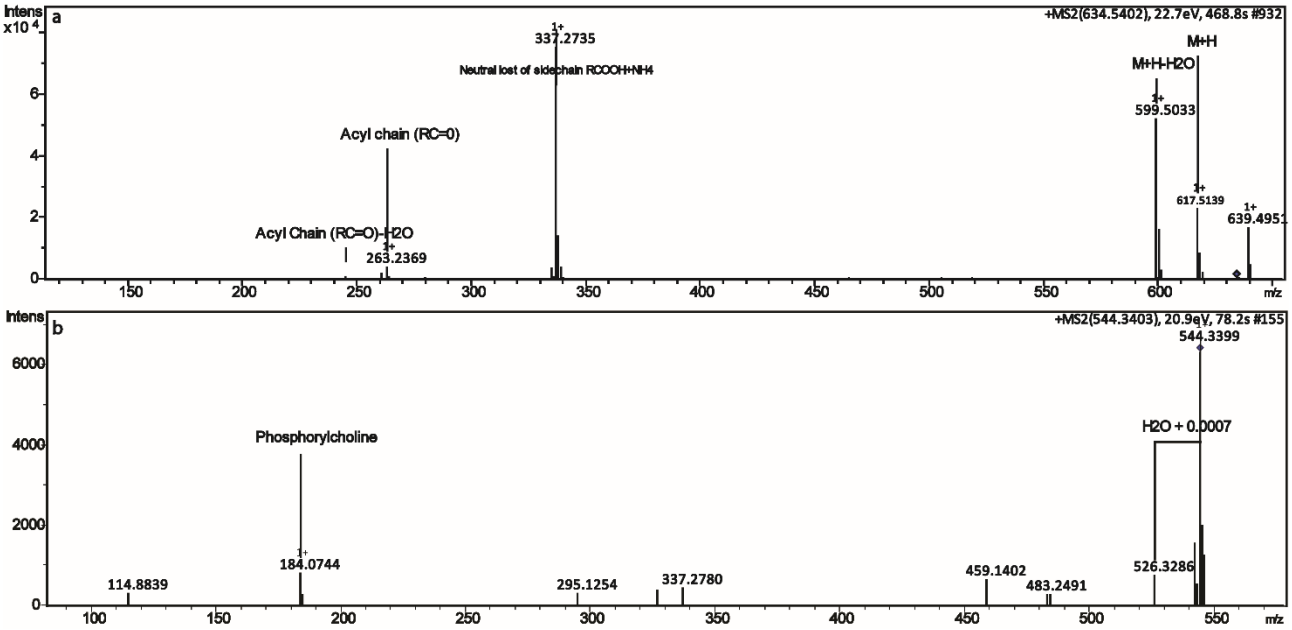

Figure 8 The MSMS pattern of diacylglycerol(18:2/18:2) and phosphatidylcholine(20:4).

The MSMS pattern of phosphatidylcholine (20:4) was identified from the MSMS pattern shown in Figure 8b. The MSMS spectrum contains a psedo-molecular ion of 544.3399 was found along with a loss of water (526.3286), lastly a fragment with the mass of 184.0744 was found. The phosphatidylcholine compounds were identified by a loss of water from the pseudo-molecular ion along with a phosphorylcholine fragment of 184.074, Figure 8b.

One metabolite in particular (from **Paper III)** was difficult to identify. A search in HMDB for the exact mass revealed three possible compounds with the same elemental composition. A standard of one of the possible compounds, cholesteryl acetate, was purchased and analyzed in the HPLC system; however, the retention time of cholesteryl acetate was not similar with the unidentified metabolite. The metabolite was therefore not identified.

5 Part V: Results and Discussion of individual projects

5.1 Method paper

LC–MS analysis of the plasma metabolome—A novel sample preparation strategy

Analysis of the plasma metabolome is a complicated task due to the large polarity and concentration range of the metabolites. For this study, the main aim was to achieve a high number of molecular features by determination of three distinct metabolite classes: phospholipids, neutral lipids and polar compounds.

Two pre-treatment approaches for the purification of plasma samples were tested. The first approach was based on protein precipitation, using three volumes of organic solvent followed by HPLC-MS analysis as described by Souverain *et al.* (Souverain et al 2004). The second approach was based on protein precipitation followed by SPE to separate the phospholipids.

Figure 8 shows a sample obtained by the first purification procedure. The chromatography showed sharp peaks from the first injection (Black chromatogram), however a second injection (Grey chromatogram) revealed a problem with precipitation of the phospholipid in the first run. The phospholipids from the first injection eluted between 300-500 s during the second HPLC-MS run resulting in high intensity peaks causing detector saturation. This might have been resolved with a longer run-time in the presence of a high-percentage organic solvent. However, poor peak shape and detector saturation might still occur.

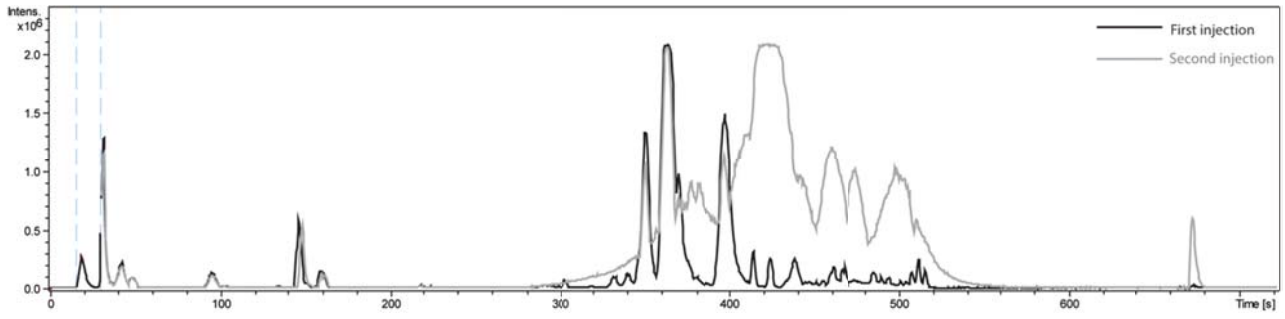

Figure 9 Protein precipitation followed by HPLC-MS. The first injection (black line) revealed a chromatogram with sharp peaks. However, upon a second injection of the same sample the chromatogram revealed high intensity peaks and detector overload (grey line). This indicated that the first sample was not fully eluted and therefore eluted in the second run.

To prevent carry-over, a second approach was tested. For this procedure, an SPE column was used to retain the phospholipids. Protein precipitation was performed using 3 volumes acetonitrile:plasma followed by centrifugation. The supernatant was placed on an SPE column and the eluate subsequently divided into two sub-fractions. This resulted in a non-polar lipid and a polar fraction; see Figure 10. The division of the sample into three gives the possibility to optimize the chromatographic conditions for each class of compounds e.g. polar compounds are best separated in a system starting with a low percentage of organic

solvent, however such conditions will result in precipitation of non-polar compounds on the column resulting in poor peak shape. Also the division into three sub-samples gives the possibility to optimize injection volumes for each class to avoid detector saturation.

Analyses of the chromatographic data using the molecular feature algorithm (Bruker Daltonics, Bremen, Germany) showed that the precipitation approach yielded 1792 molecular features, while the SPE fractionation approach yielded totally 4234 molecular features from the three fractions. Also, in contrast to the procedure based on the study from Souverain *et al.,* no carryover was observed. Hence, the SPE approach was used for the extraction of the metabolites in **Paper I-IV**.

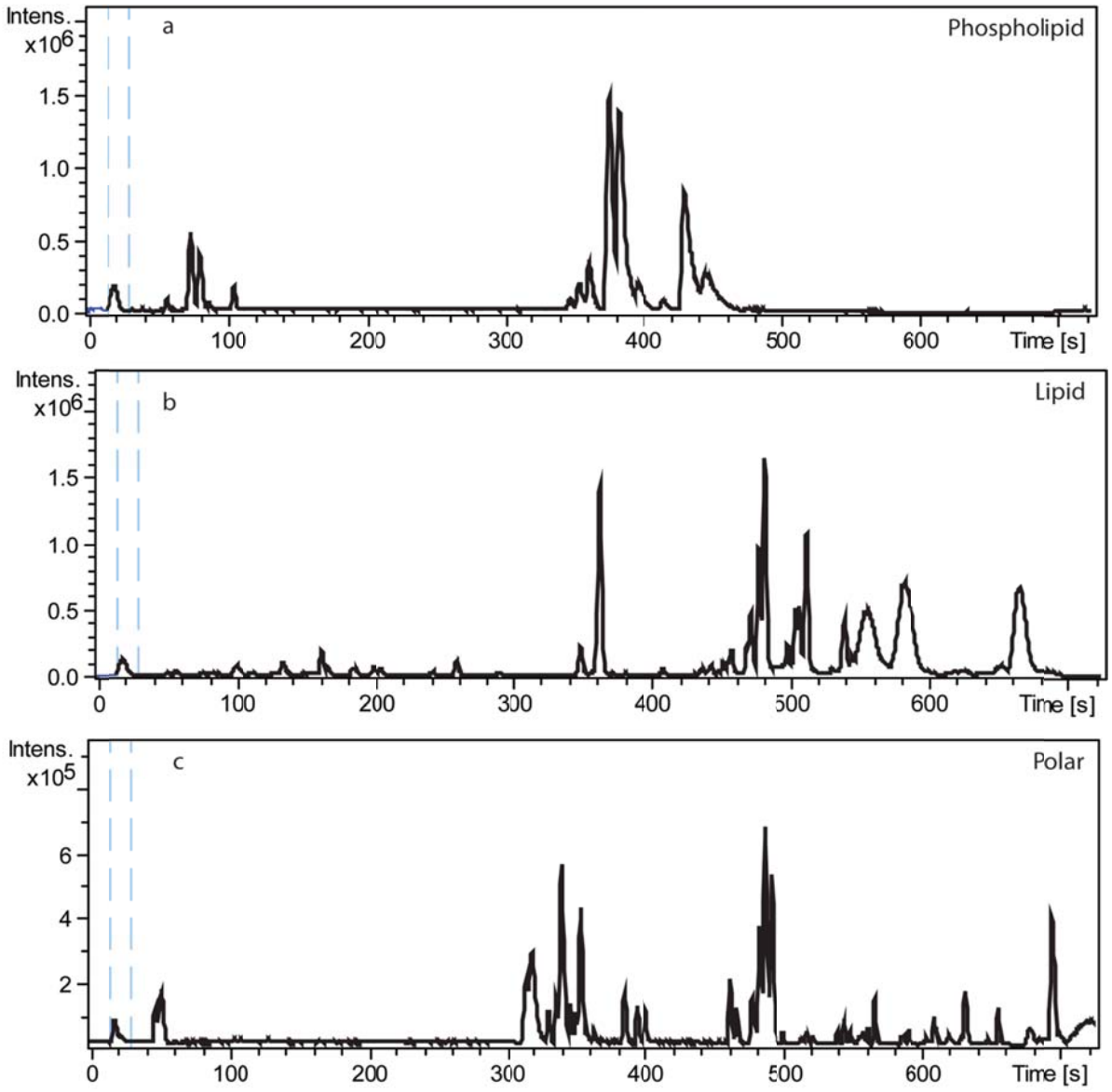

Figure 10 An example of a chromatogram using the selected purification procedure. The three fractions are a) phospholipids b) lipid fraction and c) the polar fraction. The clustering in chromatogram a) around 100 s corresponds to lyso-phospholipids while that between 350-500 second are the diacylated phospholipids.

The method is similar to that described by Tulipani *et al.* (Tulipani et al 2013), but with extraction of phospholipids for analysis since plasma phospholipid concentrations were found to be sensitive to chemical exposure. Therefore, the phospholipids were extracted from the column for analysis. In Figure 10 , the three analyzed sub-fractions are shown. The reason for having three sub-fractions was to reduce the ion suppression from both the phospholipids and the neutral lipids by optimization of injection volume and chromatography. This method was used to analyze all samples in the two studies described in the following sections; the "PFNA Papers" and "Test of the method – low dose exposure".

With the established cleanup/purification method, we identified approximately 2.5 times more molecular features compared to a traditional protein precipitation method (Souverain et al 2004). This is slightly overestimated, since the selected method will include some compounds twice as these are equilibrium distributed between the heptane and the methanol extraction solvent. It is notable that the selected method is based on a non-targeted approach, which implies less knowledge about the contents of specific compounds. For analysis of the metabolites on the LC-MS system it was found that a C8 column was the best compromise to give a good chromatographic separation of all three sub-fractions: polar, lipid and phospholipid. By using a single analytical column, all three sub-fractions could be analyzed in one sequence, saving on time and cost of the analyses.

The importance of phospholipids has been described in several studies, e.g. Psychogios *et al.* (Psychogios et al 2011) and Kim et al. (Kim et al 2011), who showed that phospholipids were affected by hepatic steatosis. Furthermore, phospholipids are believed to have signaling activity (Chen et al 2011; Zhang et al 2014). Therefore, changes in phospholipid levels may provide information on specific mechanisms and effects in mammals. Thus, we thought it important to include these compounds in the analysis.

Using the new SPE based method, three major classes of the plasma metabolites were detectable; the phospholipids, neutral lipids and the polar fraction. We detected differences in phospholipid, neutral lipid and polar fractions of the plasma (**Paper III, IV and V**). The majority of detected changes in phospholipids were phosphotidylcholine, but neutral lipids such as mono-, di- and triglycerides were also detected. Furthermore, it was possible to detect steroid hormones in the exposed animals to a certain extent (**Paper III**).

5.2 PFNA papers

Paper II + III: "Perflourononanoic acid in combination with 14 chemicals exerts low-dose mixture effects in rats" and "Exposure to Perflourononanoic acid combined with a low-dose mixture of 14 human relevant compounds disturbs the energy/lipid homeostasis"

Perfluoroalkyl acids (PFAAs) are stable, man-made perfluorinated organic molecules characterized by a fluorinated hydrophobic linear carbon chain of varying length terminated by a carboxylic acid (Klenow et al 2013). Perfluorononanoic acid (PFNA) is a PFAA with a nine-carbon backbone. The potential health concerns regarding perfluoronated compounds have arisen due to their global distribution, environmental persistence, accumulation among humans and wildlife, and extraordinarily long biological half-life (Lau et al 2007; Fang et al 2012a; Yamada et al 2014).

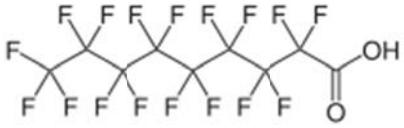

Figure 11 Structure of PFNA

Metabolomics has previously been used to analyze changes in the metabolome as a result of toxicity (Nicholson et al 1999; Robertson et al 2005; van Ravenzwaay et al 2014). The effect of high-dose exposure is well described in the toxicological literature (Nielsen et al 2010b; García-Sevillano et al 2014), however, the investigation of low-dose exposures have just recently been undertaken (Crofton et al 2005; Merhi et al 2010; Demur et al 2013). Metabolic changes are likely to be initiated already at lower exposure levels than those at which overt toxic effects are observed. This means that, at a high concentration both overt toxicity, as well as biochemical changes can occur. To evaluate if the changes in the metabolome were different upon high and low exposures, a study including high doses with overt toxicity and low doses with a more subtle toxicity (**Paper II**) was undertaken. The experiment was designed to include both conventional toxicology techniques and a systems biology approach. The study was designed to determine the endocrine disrupting properties of PFNA. To mimic a general human exposure to several chemicals simultaneously, a low dose mixture (Mix) was also included (see paper II for details on Mix). In the conventional toxicology approach, we analyzed organs and the steroid hormones. In the systems biology approach, we analyzed the mRNA levels of liver genes and the plasma metabolome. This study design allows for the scrutiny of potential responses in the metabolome to low, mid and high dose exposure. The rats were exposed to PFNA at three doses, the lowest being related to high end human exposure, while the mid and high were 20 and 400 times higher, respectively. Analysis of the blood and the liver was conducted by metabolomics and transcriptomics, respectively.

By the conventional toxicology approach, we found effects on steroid hormones and absolute liver weights. Histopathological analyses of the livers revealed hepatic steatosis indicated by increased cell sizes and a reduced cell border line. The internal doses of PFNA following the exposure regimen was conducted and showed a concentration in high dosed animals ranging from 0.54 to 0.6 mg/ml for PFNA and PFNA+Mix, respectively, while the low dose showed between 396 and 1111 ng/ml, with or without mix, similar to sixty times human exposure..

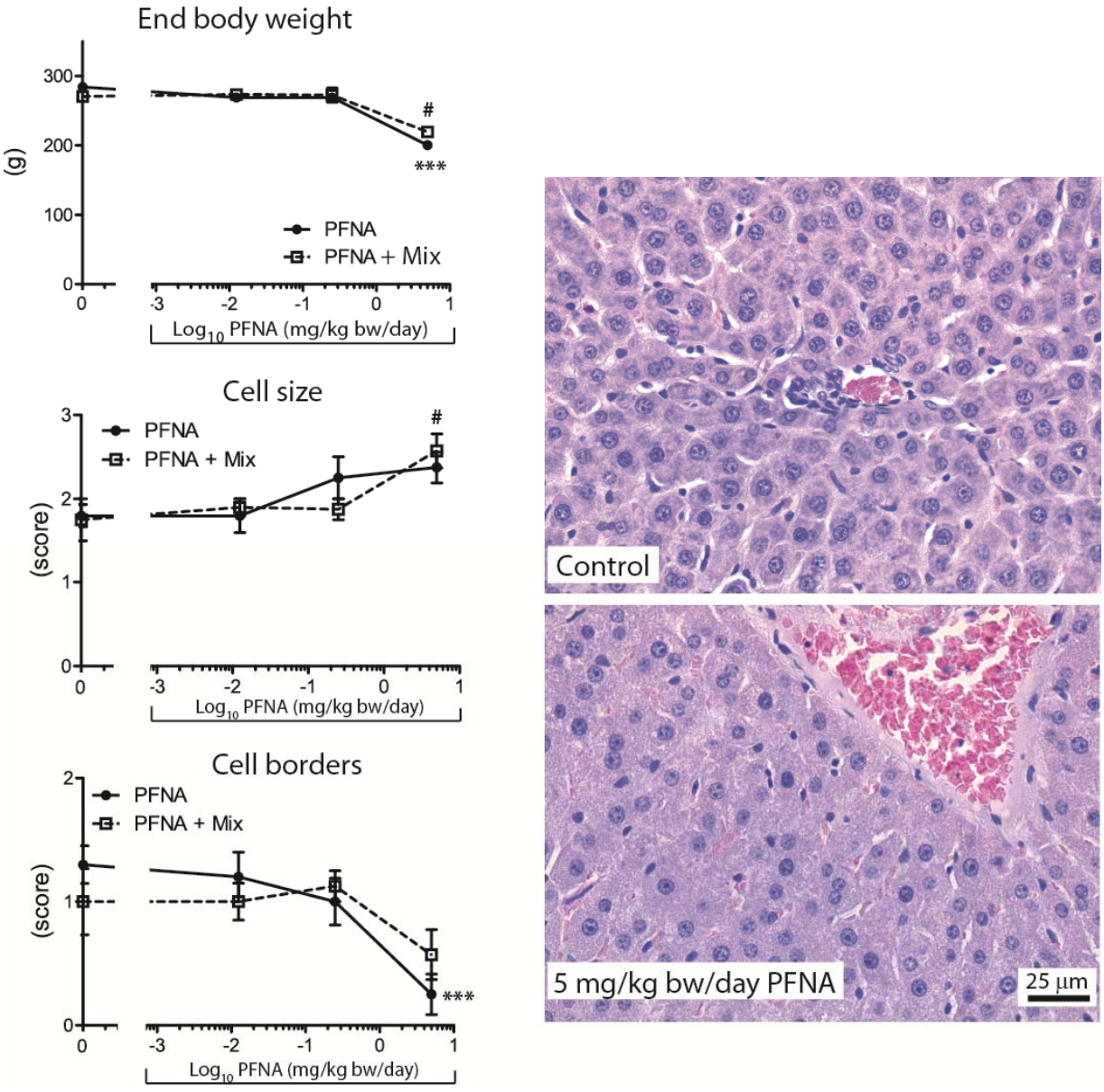

Figure 12 Physiological data from the eight groups of animals (paper II). The data show that the highest concentration of PFNA exposure lower the body weight of the animals. Furthermore, is there a change in the liver the cell size as well as the cell borders is changed upon high exposure to PFNA.

At low concentration there were only marginal effects detected by the conventional toxicological approach, although we note that an important finding was on steroid hormones with an increase in testosterone for PFNA plus Mix and an increase in corticosterone for PFNA alone (Paper II). To gain further insight into the effects of PFNA with or without Mix analysis of the plasma metabolites was applied. Furthermore, microarray gene expression analysis was used to determine the transcription of the liver gene mRNA levels.

In animals exposed to Low PFNA only, we also observed changes to the blood metabolome, albeit different to what was observed with Mix only. In the scoreplot the different exposure groups clustered separated from each other. The lipid fraction is depicted in Figure 13. There was a clear difference between Low PFNA and Mix groups, but it was not possible to positively identify the specific metabolites responsible for this separation.

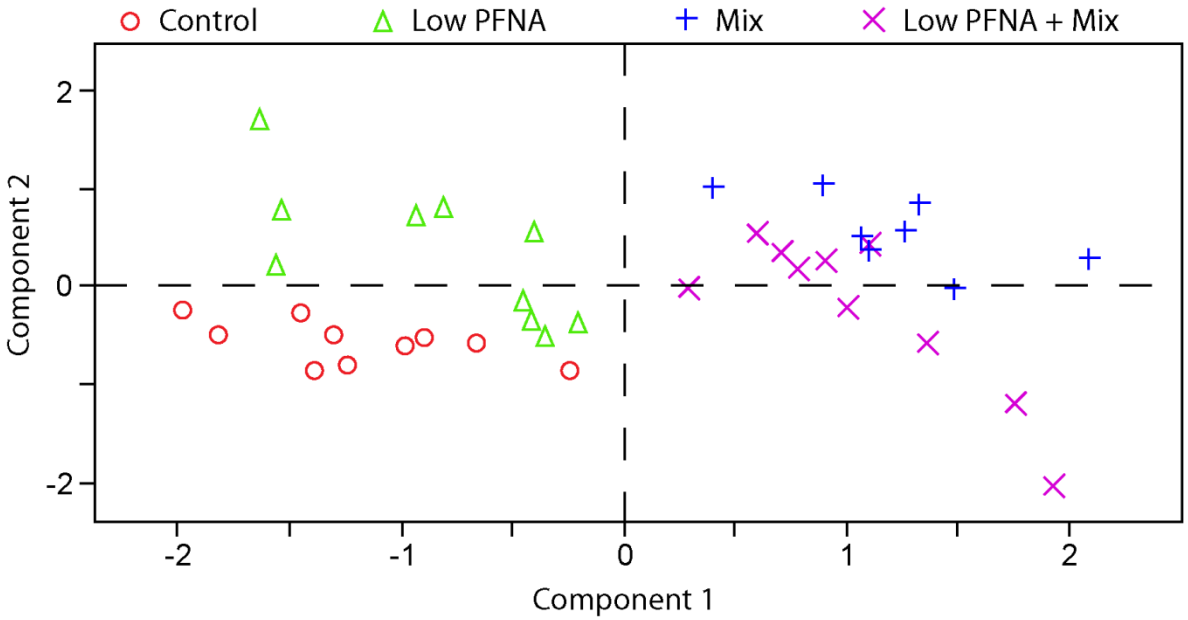

Figure 13 PLS-DA plot of the heptane fraction correlating the four groups Control (n = 10), Low PFNA (n = 10), Low PFNA + Mix (n = 10) and Mix (n = 8). The plot illustrates that Mix accounts for a major part of the variance in the dataset (Paper III) (Skov et al 2015)**.**

The plasma lipid concentration for both phospholipids and neutral lipid species were found to be lowered when the animals were exposed to mixture. The observed high impact of Low PFNA on the corticosterone in Figure 14c correlates with observations from the conventional toxicological approach employing a targeted LCMS approach (**Paper II** Fig 4) which also shows the corticosterone level to be increased at the low PFNA dose.

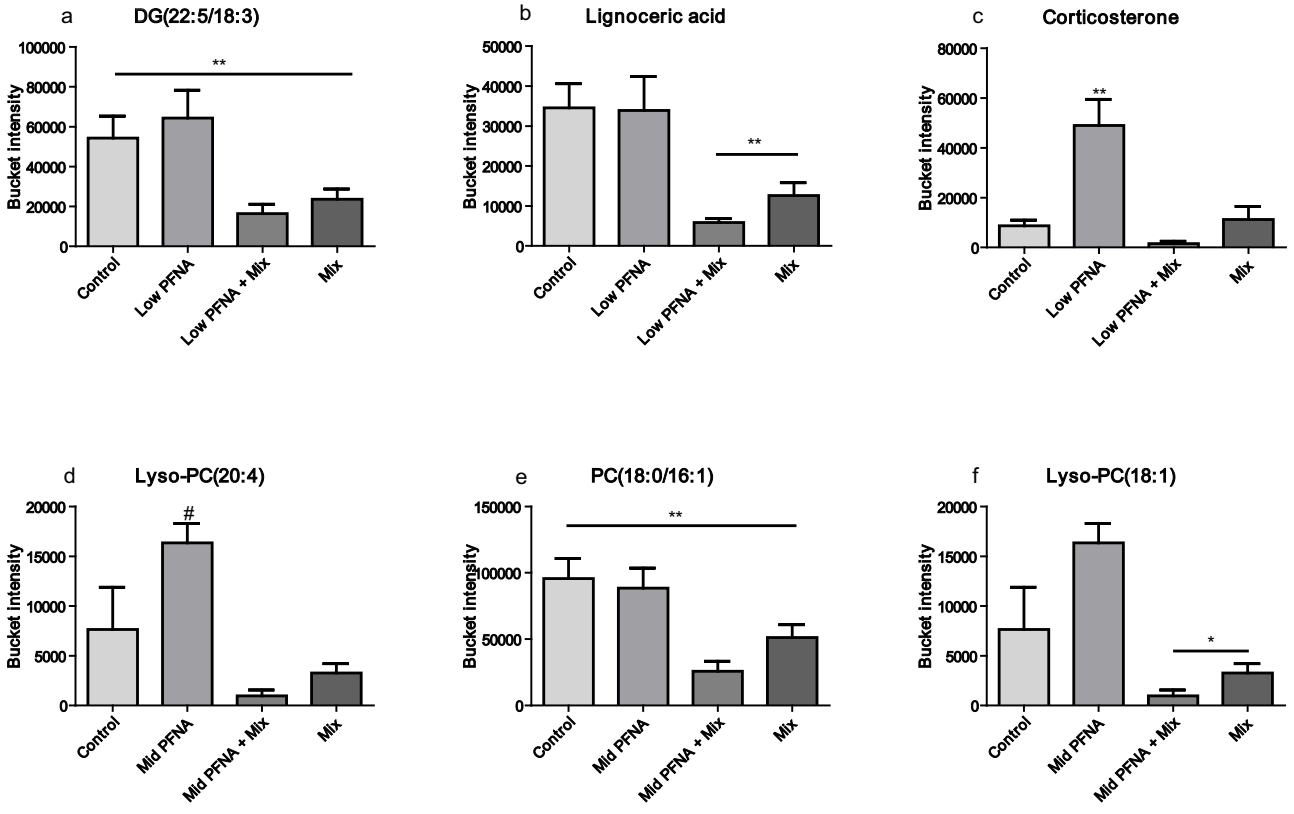

Figure 14 Representative metabolite changes in the Low PFNA + Mix group. The plotted metabolites belong to two metabolite classes; PC (phosphatidylcholine) and DG (diacylglycerol). Metabolite levels were analyzed statistically for each group by comparison of each group to control (t test). For corticosterone (c) and lyso-PC(20:4) (d), PFNA alone caused an increased metabolite level, whereas the combination of Mix and PFNA generally caused a decreased metabolite level (DG(22:5/ 18:3 (a) and PC(18:0/16:1) (e)). For lignoceric acid (b) and lyso- PC(18:1) (f) the effect by Mix + PFNA is stronger than the effect of Mix or PFNA alone. # = p-value of 0.053 (Paper III) (Skov et al 2015)**.**

Altered lipid metabolism is a common response to xenobiotic exposure (Zhang et al 2009; Karami-Mohajeri and Abdollahi 2011; Androutsopoulos et al 2013). An initial stress-response to a xenobiotic insult can increase carbohydrate metabolism to meet a changing energy requirement, subsequently met by increased lipid and protein metabolism (Karami-Mohajeri and Abdollahi 2011). The general decrease in plasma lipid levels observed in the Mix-exposed rats could reflect this situation, particularly since the exposure lasted for a prolonged period (14 days), potentially resulting in effects such as hepatic injury. From the highest exposure levels changes in the liver were found. It can be speculated that if the animals were exposed for a longer time period with mid dose of PFNA the animals might have developed macroscopic changes in the liver. Interestingly, two of our identified lysophosphatidylcholines, lyso-PC(18:2) and lyso-PC(20:4) have both been identified as potential blood biomarkers for drug-induced hepatic phospholipidosis (Saito et al 2014), and in both instances the response is reduced levels. Then, as drug-induced phospholipidosis has been associated with liver inflammation and fibrosis (Rigas et al 1986; Lewis et al 1989), it could suggest that we are observing compromised livers in our rats exposed to Mix, albeit not observed at the macroscopic level.

Based on functional annotation of genes dysregulated in the PFNA ± Mix dosing groups, fatty acid metabolism was the main biochemical function affected (Paper III Table 2). For instance, upregulated genes such as Crot, Crat, Acox1, Ehhadh, Hadha, Hadhb, Decr2, Eci2, Ech1, are all involved in peroxisomal fatty acid β-oxidation, whereas Cpt2, Slc25a20, Acad11, Acadl, Acadm, Acads, Acadvl are all involved in mitochondrial β-oxidation. Genes associated with lipid transport, fatty acid activation, and peroxisomal transport (Apoa2, Abcd3, Cd36, Slc27a2) were also upregulated. Amongst the down regulated genes was Fatty acid binding protein 5 (Fabp5). Similar effects on lipid homeostasis have been shown in other studies (Guruge et al 2006; Rosen et al 2007; Fang et al 2012b). Here, the effects on lipid metabolism seem to be mainly driven by PFNA exposure, as the effects are comparable between animals exposed to Mid PFNA only and Mid PFNA + Mix.

The mRNA levels of the liver were evaluated by micro array and it was observed that a group of receptors, the peroxisome proliferator-activated receptor (PPARs), was activated. In Figure 15, illustrating the signaling network for PPARs, transcription factors are depicted at the top of the figure while genes and phenotypic expressions are depicted below. It was found that activation of PPAR is correlated with reduced lipid concentration and increased beta-oxidation.

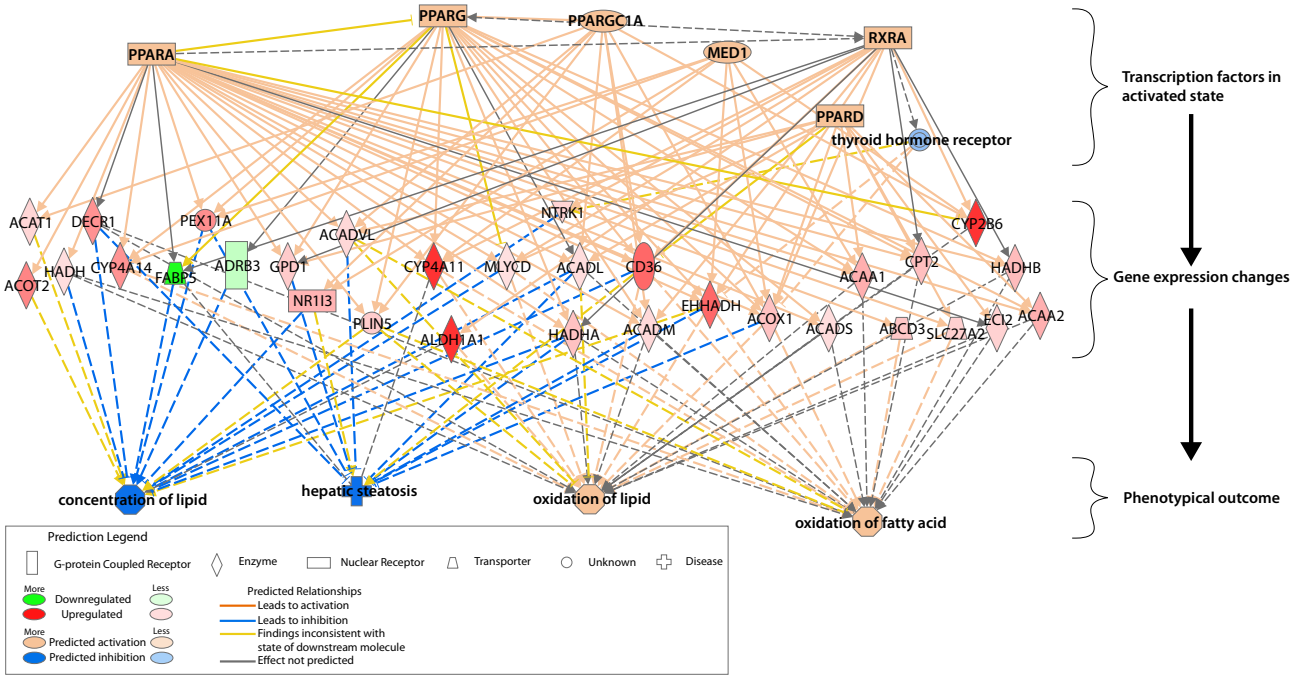

Figure 15 Illustration of a putative hepatic signaling network, from PPAR (and other transcriptional regulators) activation through regulation of gene expression to ultimately dictate phenotypic outcomes. Orange indicates predicted activation and blue indicates predicted inhibition. Shades of red and green indicate level of increased and decreased gene expression, respectively. The network was generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity (Paper III) (Skov et al 2015)**.**

PPARs are known regulators of fatty acid β-oxidation (Kanehisa and Goto 2000), and increased β-oxidation results in decreased plasma lipid concentration (Lau 2012). Then, as perfluorinated alkyl acids are known PPAR-activators (Vanden Heuvel et al 2006; Lau 2012; U. S. Environmental Protection Agency 2014), effects observed in livers from the PFNA groups could, at least in part, be explained by this regulatory pathway. Increased PPAR activation as displayed in Figure 15 could explain the effects shown at high-dose exposure. As PPAR activation leads to increased oxidation, more lipids would be present in the liver. It would be of interest to explore if macroscopic effects such as hepatic steatosis would be observed if the mid dose study was prolonged to 30 days.

NMR can provide a more precise identification of the metabolites. The plasma samples from the PFNA-exposed animals were analyzed by NMR at the Wishart Lab, Edmonton, Canada. The samples were analyzed for 43 polar compounds (**appendix III**) and no difference between control and Low PFNA was found. For the high dosed animals, seven metabolites were significantly different, at the mid dose only one metabolite, arginine, was changed.

When using the conventional toxicological approach, we found that the animals exposed to the highest concentrations developed non-alcoholic liver disease. The transcriptomics and metabolomics data suggested changes to lipid metabolism. The effects of changed lipid metabolism could be correlated with activation of PPAR as suggested by the transcriptomics data. We found that the effects were measurable at a lower level of exposure using metabolomics than what was measurable by more conventional histopathological analyses. The changes to the metabolome were less specific than a change in physiology. However, the metabolite profile of the plasma can help to understand how toxic compounds affect animals, and possibly humans, suggesting a role for systems biology in general and metabolomics in particular in future toxicological investigations into low dose effects.

5.3 Further testing of the usefulness of metabolomics in low dose toxicity experiments

Paper IV + V: "Metabolic Profiling of Rats and their Offspring Exposed to Low Doses of Bisphenol A" and "Comparison of biological effect profiles of chemical congeners using metabolite pattern determination the example of selenium nanoparticles and sodium selenite"

Bisphenol A (BPA) is an industrial chemical used to manufacture polycarbonate plastics, epoxy resins and thermal printings (Boucher et al 2014). BPA is detectable in consumer products, food, water and dust (Alonso-Magdalena et al 2008; Asahi et al 2010; Boucher et al 2014). Human exposure has been confirmed by analysis of urine, blood and tissue (Asahi et al 2010). The biomarkers of BPA is the compound itself along with the major metabolite; glucuronidated BPA (Bjerregaard et al 2007).

Selenium (Se) is an element that, in trace amounts, is essential for humans. Se is incorporated as selenocysteine, and selenium is necessary for the synthesis and catalytic function of selenoproteins such as peroxidases and reductases (Nogueira and Rocha 2011). At high doses, Se becomes toxic, exhibiting effects such as emaciation and weight loss (Benko et al 2012).

As shown above, the metabolic platform was able to detect effects even at low levels of exposure. In a follow-up study, effects of low dose exposure to BPA 25 and 250 µg/kg/day, as well as low dose exposure to selenium were examined. In the BPA study, mothers were exposed orally to the compounds; offspring indirectly via placenta and milk. For all three groups of animals, significant changes to the metabolite profile were found. The mothers exhibited decreased monoglyceride levels e.g. MG(18:0) and an increased cholesterol concentration (Paper IV). Female pups showed decreased levels of one diacylglycerol (Paper IV). The male pups showed changes to the phospholipids and diacylglycerol, as shown below, see Figure 16. Neither the mothers nor the female pups exhibited changes to plasma phospholipid concentrations.

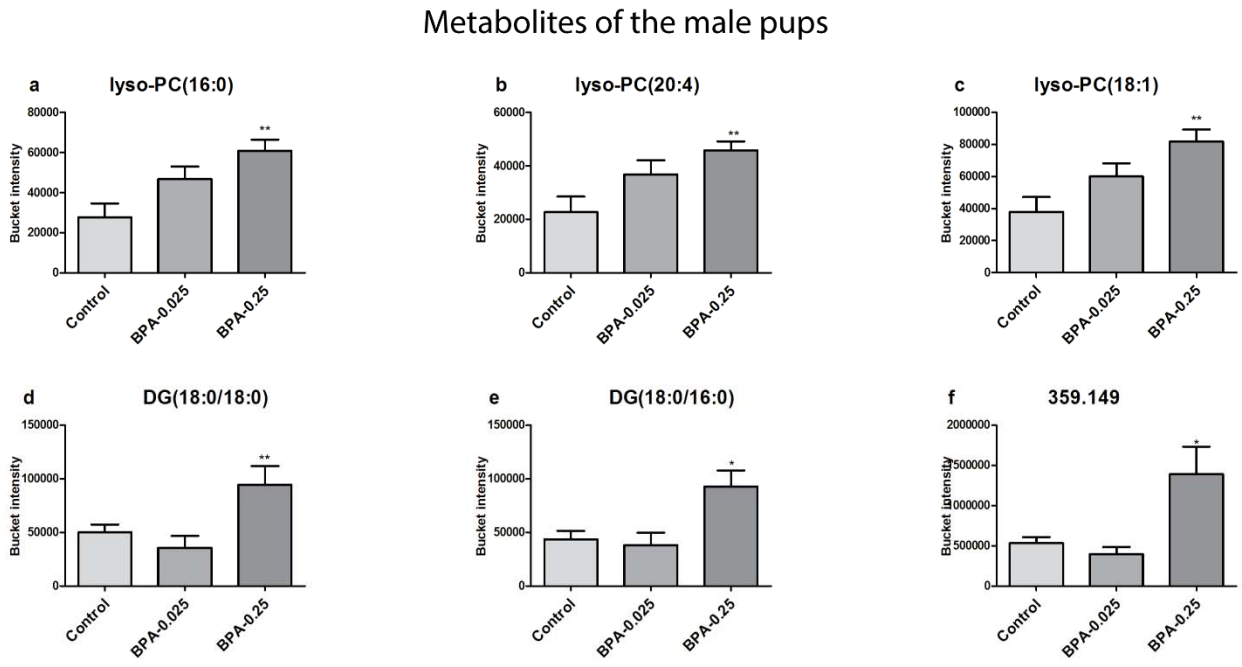

Figure 16 Significantly changed metabolites from the male pups plasma. As shown in a-c three short chain phospholipids (lyso-PCs) are significantly increased by exposure to 0.25 mg/kg/day of BPA. Furthermore, two diacylglycerols (DGs) were found to be increased by BPA exposure. One metabolite, figure f, was interesting as it was increased in the male pups while being decreased in the mother animals. The metabolite, not identified, was a pseudoion with the m/z value of 359.149 (Paper IV).

The results confirmed that it was feasible to detect changes in the metabolome upon exposure to low dose BPA. The concentrations tested in this study should, according to conventional toxicology approach, not cause any adverse effect on humans (Tyl 2013).

In the selenium study, metabolome analysis was used to examine if changes in urine metabolites upon exposure to ionic selenium were different than those exposed to nano-particle selenium. This could provide information on whether selenium nanoparticles have the same mechanism of action and toxicological potency as selenium ions. Figure 17 shows that the pattern of effects was statistically similar for the two

different selenium formulations. Together with findings for other metabolites, these findings suggest similar mechanism of action and toxicological potency of selenium nanoparticles and selenium ions (**Paper V**). Thus, this study serves to demonstrate the usefulness of metabolomics to compare effects of different congeners in terms of potency and mechanisms of action.

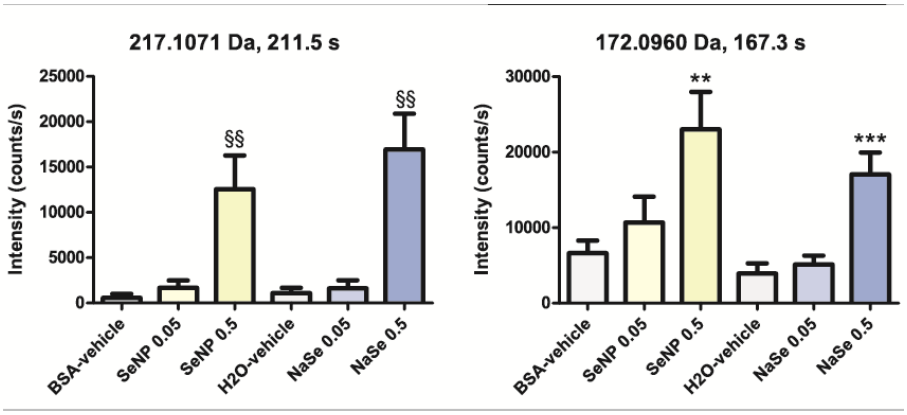

Figure 17 Plot of two pseudoions found significantly changed upon selenium exposure. As shown on the graph there are two control groups, BSA vehicle and H20 vehicle. From the intensity of the two compounds it can be seen that there is a similar level of the two metabolites in the animals exposed to high concentration of selenium from both the ionic and the nanoparticle sources (Paper V).

Multiple studies have suggested effects on the plasma metabolome exposed to a low dose of chemicals (Vandenberg et al 2012; Chen et al 2014). The data evaluated in this section are from low dose exposure studies of PFNA, BPA and selenium (**Paper I, III and V**). The analysis leads to questions related to the relevance of such studies, which will be discussed below.

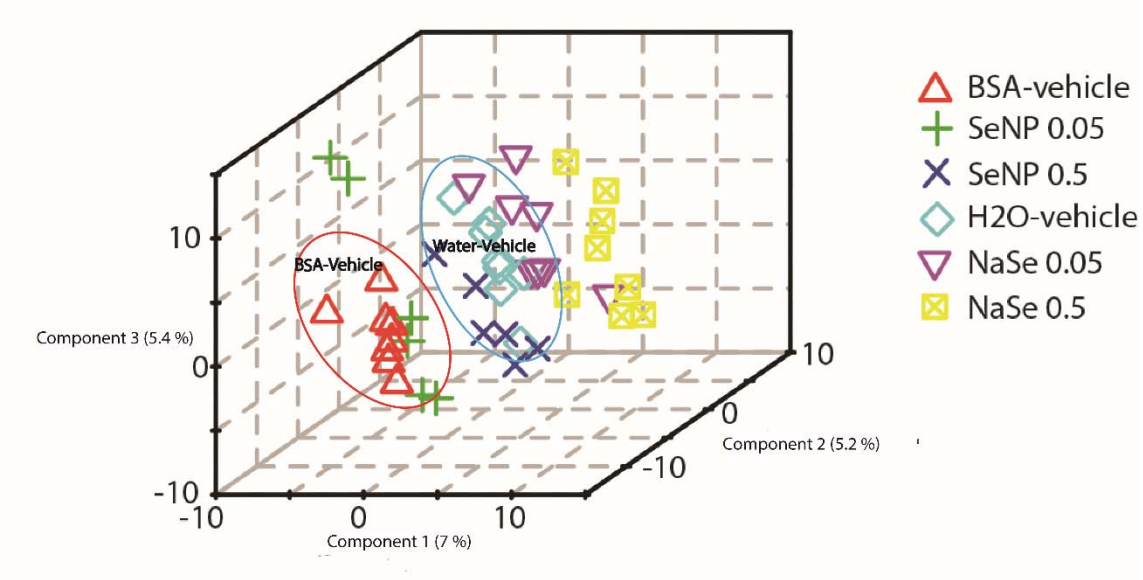

Figure 18 3D PLS-DA from a Selenium experiment. The water and BSA vehicle are clustered differently in the 3D PLS-DA plot (Paper V).

Part VI: Discussion

The increased focus on metabolic changes in the research community has widened the application scope of the metabolomics approach. In toxicology, it has been used since the late 1980's and is now used in disciplines such as pharmaceutical development, food chemistry and environmental research (Mushtaq et al 2014). According to Johnson *et al.* the metabolome is central for the exploration of toxic effects because metabolites are involved in the mechanisms underlying most effects in the mammalian body (Johnson et al 2011). Metabolomics approaches have been applied to a variety of organisms e.g. yeast, fungi, rodents and humans. In humans, for the analysis of for instance urine, blood and tissue samples (Souverain et al 2004; Smedsgaard and Nielsen 2005; Bajad et al 2006; Wishart 2008; Psychogios et al 2011; Tulipani et al 2013).

In the current study, metabolomics was used to investigate its usefulness in toxicology. It was to detect changes in the plasma metabolome upon exposure of rats to low concentration of toxic chemicals. The observed changes were mainly in the non-polar fraction of the plasma metabolome, particularly phosphotidylcholine and glycerides, discussed in section 6.1. It can be questioned if an alternative method could have been used for the study of the polar fraction to identify changes. This will be addressed in section 6.2. Several interesting effects were found at low dose exposure; however the question is whether these were adverse. This will be addressed in section 6.3.

6.1 Observed changes in the lipid fraction

In the current study, we observed multiple effects in the plasma lipid fraction by metabolomics (**Paper II, IV and V**). In recent years, changes in the lipid metabolome has received increasing attention (Ikeda et al 2009; Chen et al 2011). It has been suggested that lipids are not only part of energy storage, but also affects signaling: For example, phospholipids are known regulators of cellular death (Saito et al 2014). Analysis of the lipidome has a wide range of application e.g. analysis of cholesterol and triacylglycerol (TAG) levels in obesity patients (Adams and Lewis 1982; Seidlová-Wuttke et al 2005), to analyses focusing on toxic chemicals such as arsenic. Several studies have been performed that focus on changes in the lipid profile (Funk 2001; Sandra et al 2010; Ferreiro-Vera 2012; Liu et al 2013; Choi et al 2014; García-Sevillano et al 2014) and include phospholipid and neutral lipid analyses (Chen et al 2011; Liu et al 2013). The method performed well and yielded information regarding phospholipids and neutral lipids, as well as cholesterol. Moreover, a correlation between a low-dose exposure of environmental chemicals and effects on the metabolome was revealed (**Paper III and IV**).

In the BPA experiment we observe changes in neutral lipid concentrations; e.g. MG(18:0) and MG(16:0) (**Paper IV**) and in DG(18:1/18:3) and DG(18:2/16:0) as discussed in **Paper II**. This highlights the need for understanding what impact a change in neutral lipids has on physiology when evaluating metabolism. Furthermore, increased short chain phospholipid levels were observed for the male pups at low-dose exposure levels.

A comparison between the study conducted by Alonso-Malanga *et al.* and our BPA study (**Paper IV**) suggests that a low-dose exposure of BPA can interfere with insulin production, resulting in increased glucose uptake (Alonso-Magdalena et al 2008). Our results suggest a specific mode of action involving changes in the lipodome (**Paper IV**). This might be a contributing factor, alongside a general increase in obesity, for the steady increase of life-style diseases in the Western world, including decreased semen quality in European populations (Skakkebaek et al 2006). Lui *et al.* (Liu et al 2013) show that the phospholipid composition is changed in type 2 diabetes patients, which we also observed in male pups. Our results do not offer a specific answer to the question regarding the impact of toxic chemicals on life-style disease. But it shows that the current method can detect changes in the metabolome even at low-dose exposure, which may serve as a tool in the future diagnosis and investigation of these diseases.

6.2 Instrumentation

UHPLC-qTOF-MS has been used throughout this project to identify metabolites based on mass and MS/MS patterns. In the PFNA study (**Paper II**) we found that UHPLC-qTOF-MS, combined with the sampling strategy (described above), enabled measurements of changes in the rat metabolome. By using two different solvent systems for the UHPLC protocol (a hydrophobic and a hydrophilic solvent; see Materials and Methods), we obtained better separation of the metabolites and a reduced ion suppression compared to the protein precipitation method (**Paper I**). In general, the method identified a wide range of non-polar compounds with sharp chromatographic peaks. Regarding the separation of high molecular weight phospholipids, however, peak broadening complicated the identification process (**Paper I**). The method did not provide separation of polar compounds such as amino acids or urea. This can be achieved by addition of a HILIC column and a gradient system for separating the polar compounds (Bajad et al 2006; Boudah et al 2014). In the present project (**Paper II**), NMR was used to identify concentration changes in polar metabolites, but no significant changes were found at low dose exposure. At higher doses, the method revealed changes in 7 out of 43 analyzed metabolites. For analysis of the polar fraction, others have successfully used NMR to determine differences in the metabolome (Wishart 2008; Psychogios et al 2011; Zeng et al 2013). CE-MS and NMR techniques have been used to study changes in the plasma metabolome following exposure to toxic environmental chemicals such as BPA (Xu and Xu 2013).

The interest in the non-polar part of the metabolome has recently been highlighted (Taguchi et al 2005; Takatera et al 2006; Sandra et al 2010; Ferreiro-Vera 2012). Choi *et al.* (Choi et al 2014) have developed a method based on a mixture of equal amounts of isopropanol and acetonitrile in the solvent system to improve the resolution and to provide a better separation of the phospholipids. This, in combination with a lower percentage organic solvent in the starting condition of the hydrophobic gradient system, might enhance the chromatography of the lipidome.

Further improvement of the present procedure could be to combine it with other analytical instruments such as gas chromatography. This has the advantage of being able to analyze the polar metabolites by GC-MS. Furthermore, the identification process using GC-MS can be simplified, as library searches identifying the metabolites are possible (Fiehn 2001; Fiehn 2002). Multiple studies have now used a combination of these techniques to observe changes in the polar fraction of the plasma metabolome following exposure to toxic chemicals (Bajad et al 2006; Sangster et al 2006; Bajad and Shulaev 2007; Lu et al 2008; Boudah et al 2014) (Xu and Xu 2013). By using similar approaches, we most likely would have observed further changes to the polar fraction. However, the method would have been more time consuming.

6.3 Low dose exposure: adverse or biochemical effect?

The aim of this part of the work was to investigate whether a metabolomics approach could be applied to detect changes in the plasma metabolome of rats given low doses. The study was performed by comparing a metabolome analysis with a conventional toxicity approach of rats exposed to either low or high doses of chemicals (**Paper II and III**). It was found that metabolome analysis, for some parameters, could detect effects similar to those using conventional toxicology protocols e.g. an increase in corticosterone. To further investigate low dose exposure, two additional experiments were conducted; exposure of BPA or selenium.

One major question following on from these studies, were if the observed effects were transient (that is, an acute but reversible physiological response to chemical insult), or adverse (as in prolonged compromised physiology caused by chemical insult)?

A toxic insult to an organism will likely cause a metabolome change, which is also seen where physiological effects are not observed. In the Selenium study (**Paper V**), a difference in the PLS-DA plots between the clustering of bovine serum albumin controls and the water controls was observed (see Figure 18). This indicates that even low dose exposure to a non-toxic compound can result in measurable effects in the metabolome. Changes in plasma metabolites can also be used to compare mechanisms of action and effect-levels of different congeners, although no definitive identification is obtained (Paper V). In paper V,

we conclude that it is highly likely that two different formulations of Se (nanoparticles and ions) have similar mechanisms of action, although we could not specify the identities of all the significantly changed metabolites (**Paper V**).

The conventional toxicological approach is to establish dose levels at which compounds exert adverse effects. In traditional studies, pathological changes in clinical appearances or in tissues are signs of adverse effects (Nielsen et al 2010a). Metabolome analysis enables the determination of changes to metabolite concentrations even at low-dose exposures. The changes can then be linked to pathological observations or potential adverse effects. Metabolomics approaches have previously been used to reveal effects in animals exposed to toxic chemicals (German et al 2005; Shulaev 2006; Robertson et al 2011; Johnson et al 2012). Ravenzwaays *et al.* concluded that the metabolomics and the conventional approach display "nearly equal sensitivity toward determination of NOAEL"; for one case a reduced body weight was observed for both sexes, whereas a change in metabolite levels was observed in females, but not males (van Ravenzwaay et al 2014).

From the results obtained in this thesis, there is no clear answer as to whether low doses of the specific chemicals affect metabolism to cause adverse effects. Although, the effect observed for PFNA on corticosterone levels could be interpreted as an adverse effect. This, however, does not preclude that, for some chemicals, the use of systems biology in general and metabolome analysis in particular will yield information that can be extrapolated to adverse effects, e.g. effects on cholesterol metabolism or sets of biomarkers indicating liver disease. Also, the effects observed in the metabolome following low-dose exposure will increase our understanding of how environmental chemicals affect rats, and possibly humans. In the future, with further advances in systems biology, we expect metabolomics to contribute significantly towards toxicology testing of whole organisms. Information obtained from metabolomics will assist in the understanding of the mode of action of toxic chemicals. Many studies have been performed analyzing the changes in the metabolome upon exposure to toxic chemicals. Changes in the metabolome reflects the mode of action of a toxic compound and the pattern of changes metabolites is a marker for the toxic effect e.g. hepatotoxicity. Identification of markers of various toxic effects from animal studies will in the future provide a database which can be used for screening of chemicals for specific toxic effects.

7 Part VII: Conclusion

The aim of this thesis was to use metabolomics to study effect-outcomes in toxicology experiments, particularly at low dose exposure. A method was developed that allows for analysis of plasma from rats having been exposed to low doses of toxic compounds. Results obtained from this method were subsequently compared to results obtained through conventional toxicology protocols. Finally, the method was used to suggest mode of action of toxic chemicals.

The analytical method was based on SPE sub-fractioning of the metabolome into a phospholipid, a lipid and a polar fraction followed by UHPLC-qTOF analysis. The applicability of the method was demonstrated by showing changes to the metabolome of animals having been administered a chemical dose comparable with human exposure of the endocrine disruptor PFNA (**Paper I**). Nevertheless, there is still room for improvements to the method, for instance changing the phospholipid solvent and running the polar fraction in HILIC mode.

To further validate the method, comparisons to a conventional toxicology approach were performed. The observed effect a low dose of PFNA had on corticosterone correlated with observations from the conventional toxicology approach using a targeted LCMS approach (**Paper II and III**).

To investigate the potential impact of low dose exposure, experiments with PFNA, PFNA+Mix, BPA and selenium were performed in rats. In the PFNA ± Mix exposure experiment, the addition of Mix resulted in a significantly different metabolite profile with decreased levels of neutral lipids and phospholipids (**Paper III**). In the BPA experiment, the rat mothers showed a decreased level of MG(16:0) and MG(18:0), while the male pups showed increased levels of lyso-phospholipids (**Paper IV**). The selenium experiment showed that ionic selenium and nano-particle selenium induced similar changes in rat urine, which indicates a minor importance of the ionization state of selenium (**Paper V**). In all three experiments changes were observed following low-dose exposures (**Paper III, IV and V**).

An encouraging finding was that metabolomics assisted in suggesting possible modes of action for PFNA and BPA toxicity. PFNA was suggested to induce hepatic steatosis (**Paper III**), while BPA changed plasma cholesterol and phospholipid concentrations (**Paper IV**).

We observed that a mixture of 14 chemicals at high-end human exposure levels (Mix), low- to mid-range doses of PFNA, or a combination of the two, had the potential to alter the blood plasma levels of diacylglycerols, PCs, and cholesterol derivatives, mimicking some of the features of metabolic syndrome. These effects can also suggest an inflammatory response, but in either case warrant further investigations.

We also observed more subtle changes to the liver transcriptome following Mid PFNA exposure irrespective of Mix, suggesting that the hepatic effects were driven by PFNA. Transcriptomics analysis suggests increased PPAR activation, resulting in lowered lipid concentration and increased β-oxidation. Hence, this study demonstrates that low-dose exposure to chemical mixtures can affect the metabolome and cause disturbed lipid homeostasis, and that 'omics' approaches are powerful tools to detect smaller changes not readily observable at the macroscopic level.

The BPA experiment revealed a change to cholesterol levels in the mothers and a general change to the lipidome, which could be related to effects due to higher levels of insulin in the plasma; in turn resulting in an increased biosynthesis of triacylglycerol and a decrease in mono- and diacylglycerols in the plasma.

Using a metabolomics approach reveals changes to the metabolome which could not be foreseen using regular toxicological approaches. We applied a new protocol which can improve the understanding of toxic compounds and assist in toxicology-testing to identify possible mechanism of actions. Furthermore, the metabolomics approach can help in identifying specific pathways or systemic changes caused by a toxic chemical. The metabolite changes in both the PFNA and BPA papers are very interesting, but the changes might not give the full picture of how the respective compounds act. From this study, we show that an metabolomics approach can be used to increase our understanding of how toxic chemicals affect physiological parameters. It can be discussed if metabolomics can be used as a "stand-alone" approach, but we believe that it is important to use this tool as it adds another dimension to that of conventional toxicology testing.

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Appendix

Appedix I: Exposure levels

PFNA experiment (14 days)	
Control (n08)	
Low PFNA ± Mix (n=10)	0.0125 mg/kg/day ± Mix
Mid PFNA ± Mix (n=8)	0.25 mg/kg/day ± Mix
High PFNA ± Mix (n=8)	5 mg/kg/day ± Mix
Mix (n=8)	Mix

Mix:

CAS registry number	Chemical name	Source/use	Ratio in mixture (weight)	Rat dose (mg/kg bw/day)
7380-40-7	bergamottin	grapefruit constituent	0.08	0.2
59870-68-7	glabridin	liquorice constituent	0.12	0.3
80-05-7	bisphenol A	plastic additive	0.004	0.01
94-26-8	butyl paraben	preservative	0.21	0.52
84-74-2	dibutylphtalate (DBP)	plasticizer	0.02	0.06
117-81-7	bis(2-ethylhexyl)phthalate (DEHP)	plasticizer	0.03	0.09
36861-47-9	4-methylbenzylidene camphor (4-MBC)	sun filter	0.15	0.38
5466-77-3	2-Ethylhexyl-4-methoxycinnamate (OMC)	sun filter	0.27	0.68
72-55-9	dichlorodiphenyldichloroethylene (p,p´-DDE)	pesticide	0.002	0.006
133855-98-8	epoxiconazole	pesticide	0.02	0.05

330-55-2	linuron	pesticide	0.002	0.004
67747-09-5	prochloraz	pesticide	0.025	0.06
32809-16-8	procymidone	pesticide	0.035	0.09
50471-44-8	vinclozolin	pesticide	0.021	0.05

Bisphenol A (During pregnacy + 22 day after birth)	
Low BPA	0.025 mg/kg/day
High BPA	0.25 mg/kg/day
Data not analysed by metabolomics	*Data not analysed by metabolomics*
BPA 5	*5 mg/kg/day*
BPA 50	*50 mg/kg/day*

Selene (14 days)	
Vehicle-BSA control (n=8)	4 g/L
Selenium nanoparticles (n=6)	0.05 mg/kg
Selenium nanoparticles (n=6)	0.5 mg/kg
Vehicle H2O (n=8)	
Na-selenite (n=8)	0.05 mg/kg
Na-selenite (n=8)	0.5 mg/kg

Appendix II: Mass spectrometer stats

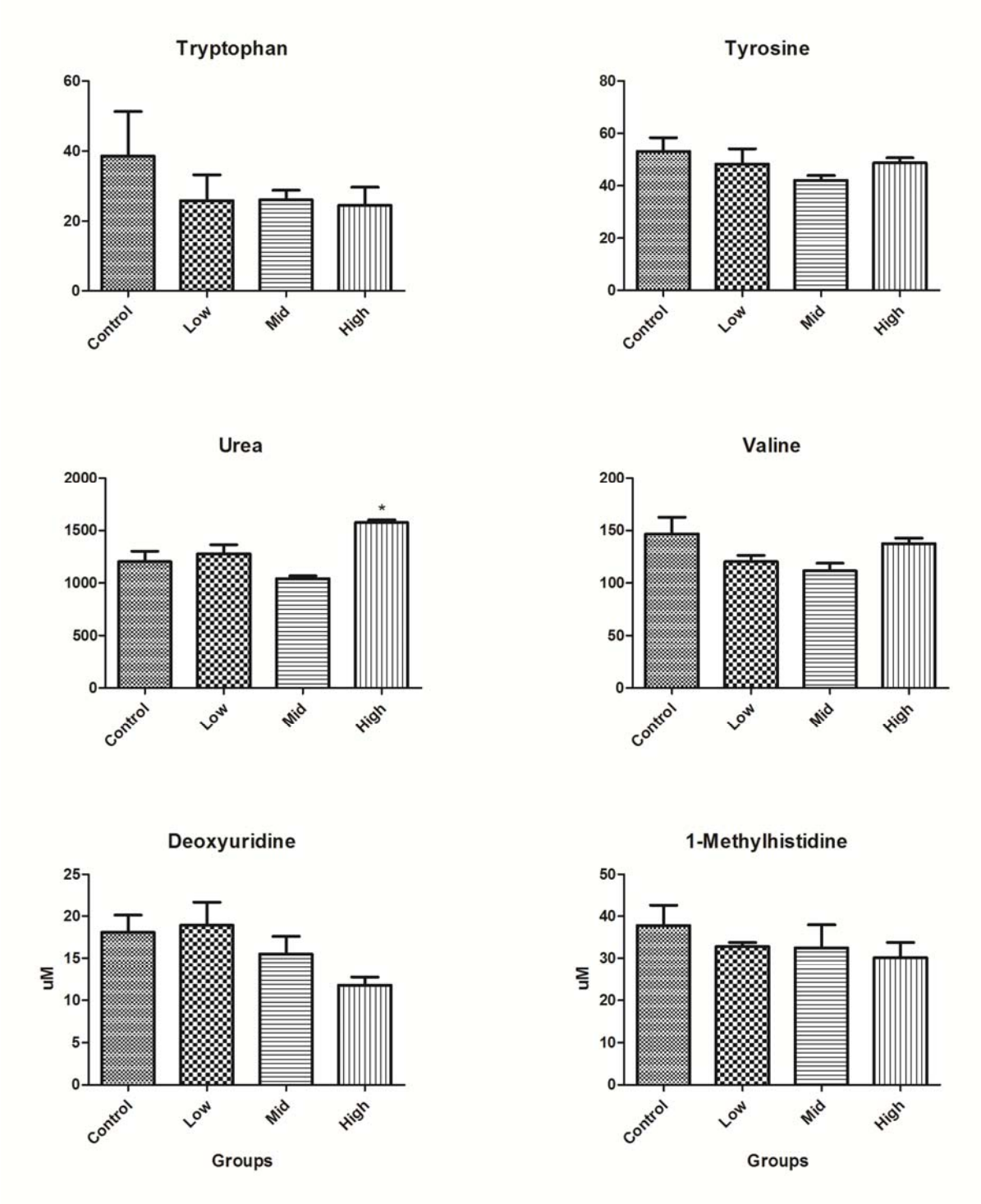
Ion source settings	
Nebulizer	2 l/min
Drying gas	10 l/min
Dry gas temp	200^{O}C
Capillary voltage	4500 V
Scan range and time	
Scan range	50-1100 m/z
Scan time	2 Hz
MSMS settings	
"Auto MSMS"	MSMS every second scan of 3 top intensity peaks Collision energy was based on the mass of the compound a ramping range of 10-30 eV for mass 100-1000 Da
Calibration	
Sodium formate	Time window (0.2-0.4)
Hexakisperflouretoxyphophazene	Evaporation from sponge in ion source

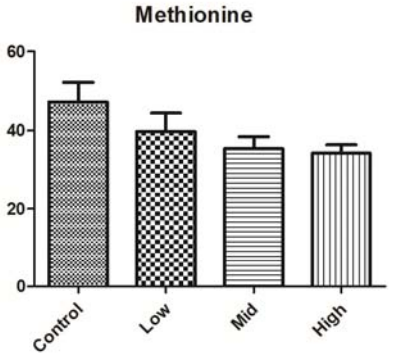
NMR setting

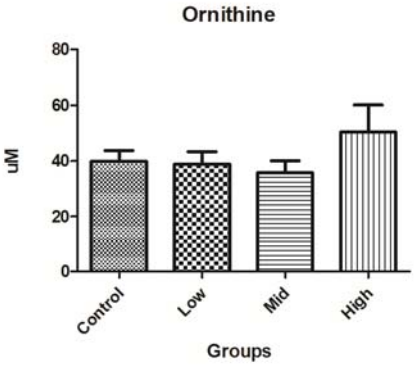
H-NMR spectra were collected on a either a 500 MHz Inova (Varian Inc., Palo Alto, CA) spectrometer using the first transient of the tnnoesypresaturation pulse sequence. The resulting 1 H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 6.0 (Chenomx Inc., Edmonton, AB), as previously described .

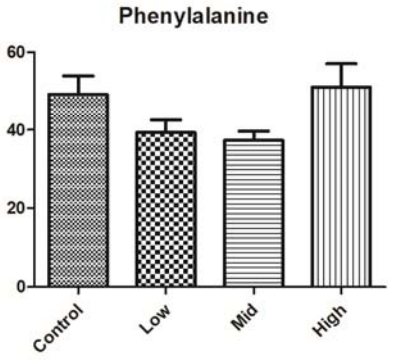
“Prior to spectral analysis, all FIDs were zero-filled to 64k data points, and a line broadening of 0.5 Hz was applied. The methyl singlet of the buffer constituent DSS served as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All 1H NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 4.6 (Chenomx Inc., Edmonton, AB). The Chenomx NMR Suite software allows for qualitative and quantitative analysis of an NMR spectrum by “fitting” spectral signatures from an internal database of reference spectra to the full NMR spectrum [22]. Specifically, the spectral fitting for each metabolite was done using the standard Chenomx 500 MHz (pH 6–8) metabolite library, with a set of additional compound signatures (1,5-anhydrosorbitol, dimethyl sulfone, 2-oxoisovalerate, 3-hydroxyisobutyrate) that were added in-house. It has been previously shown that this fitting procedure provides absolute concentration accuracies of 90% or better.” (Wishart et al 2008)	

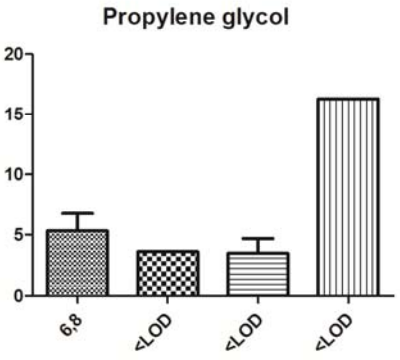
Appendix III: NMR data




Methionine
60
40
20
0
Control
Low
Mid
High


Ornithine
80
60
40
20
0
uM
Control
Low
Mid
High
Groups


Phenylalanine
60
40
20
0
Control
Low
Mid
High


Propylene glycol
20
15
10
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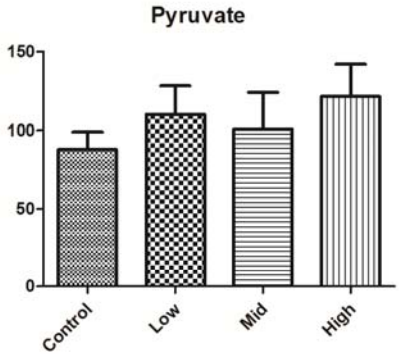
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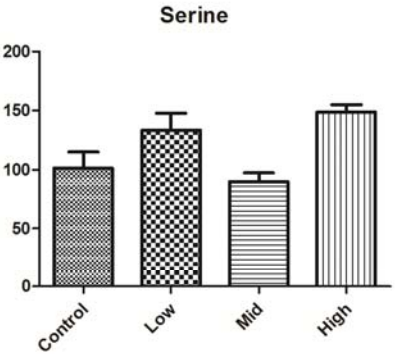
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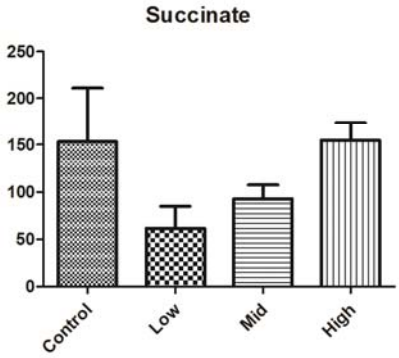
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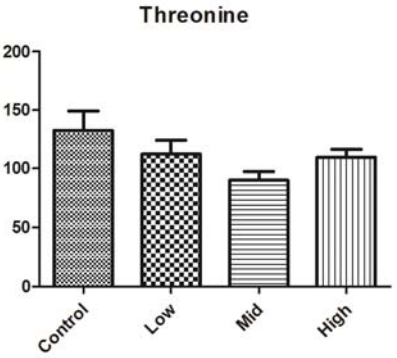
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Mid
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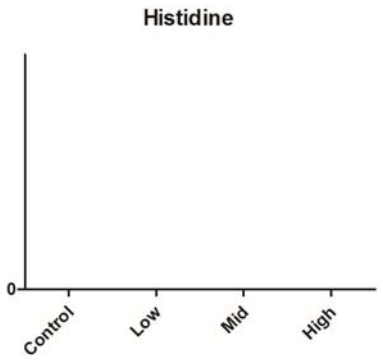
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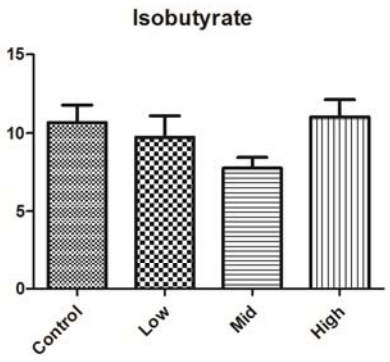
Isobutyrate
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Control
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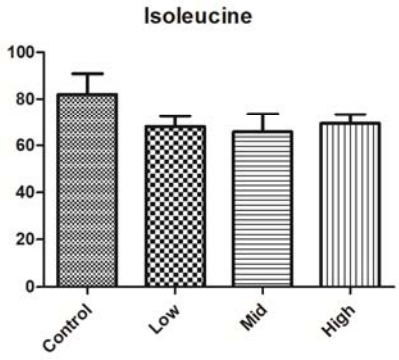
Mid
High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Mid
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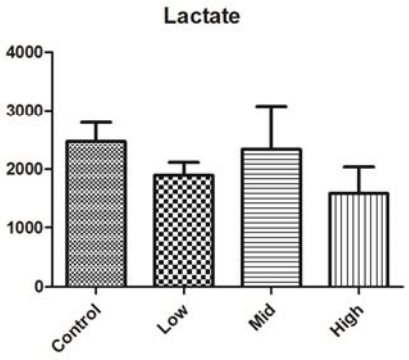
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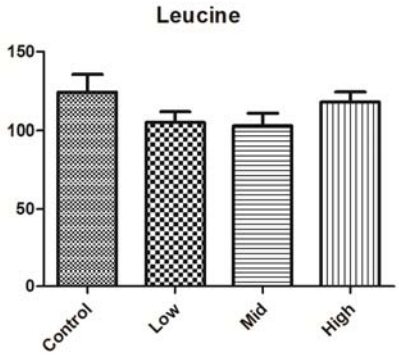
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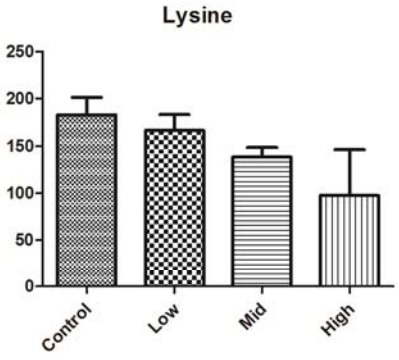
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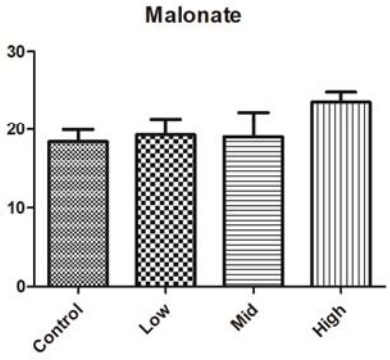
Mid
High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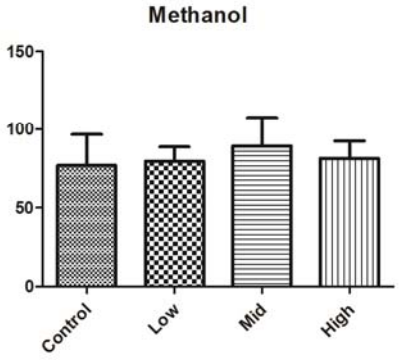
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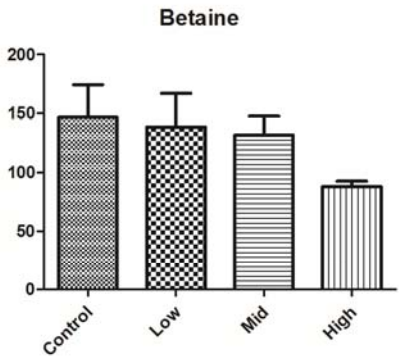
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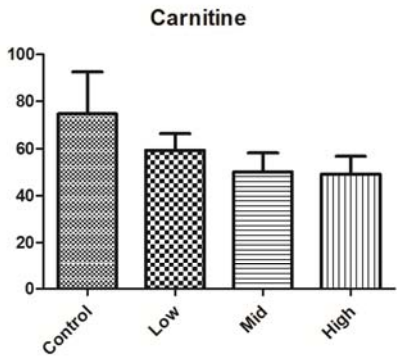
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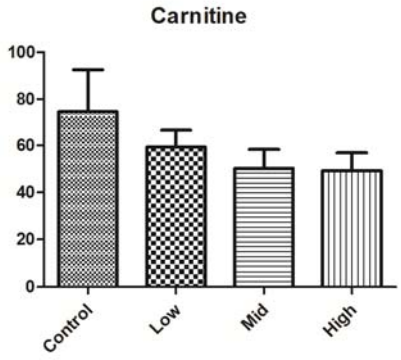
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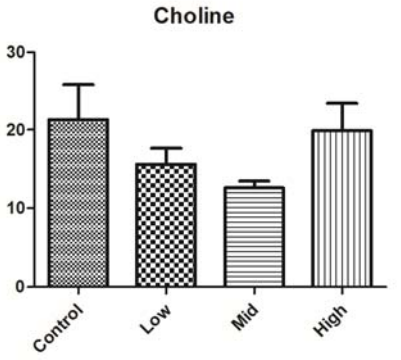
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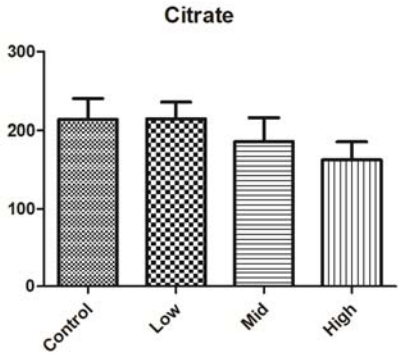
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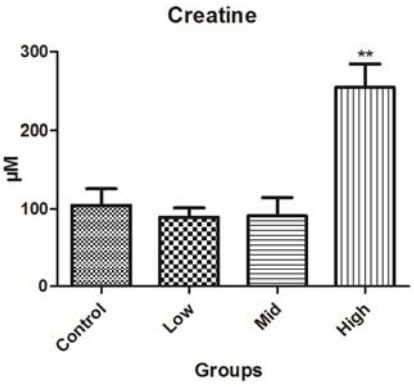
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200
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0
μM
**
Control
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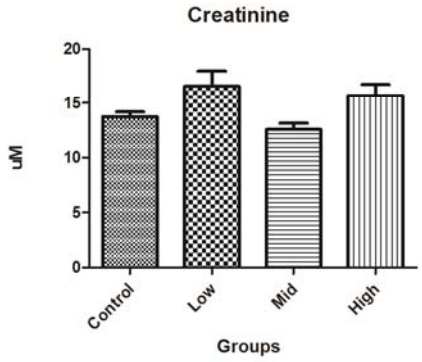
Mid
High
Groups


Creatinine
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Control
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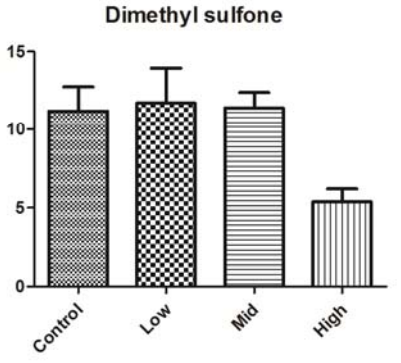
Groups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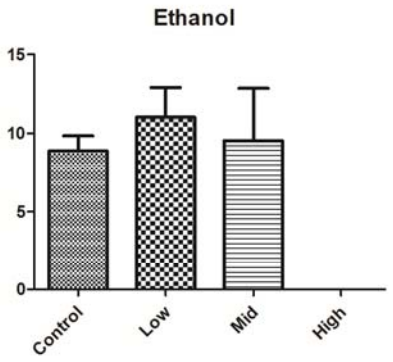
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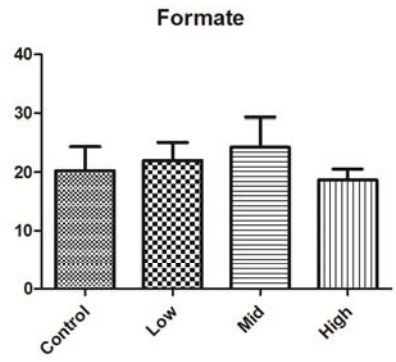
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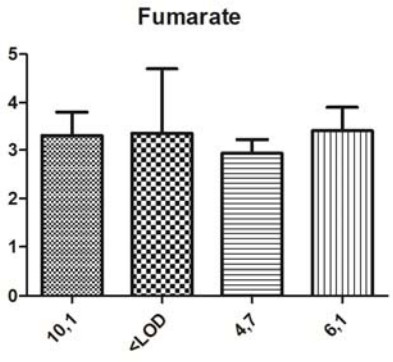
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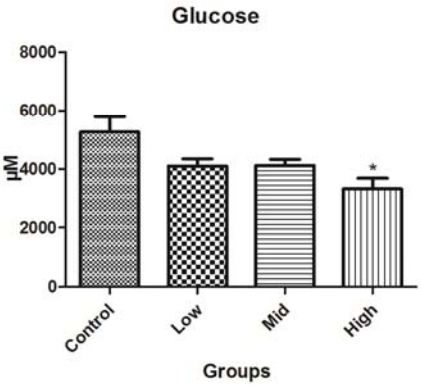
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4000
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µM
*
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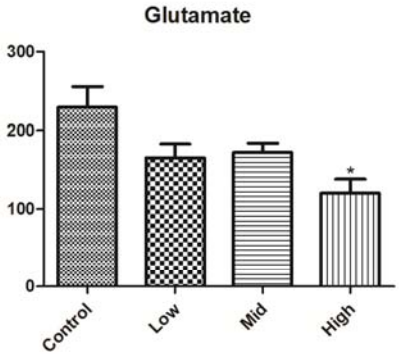
Low
Mid
High
Groups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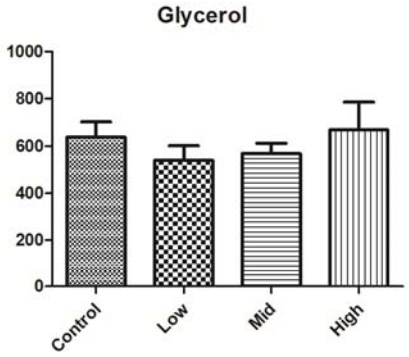
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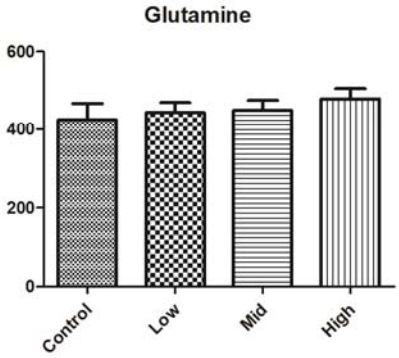
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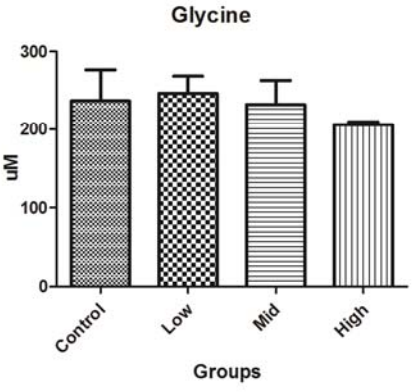
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Control
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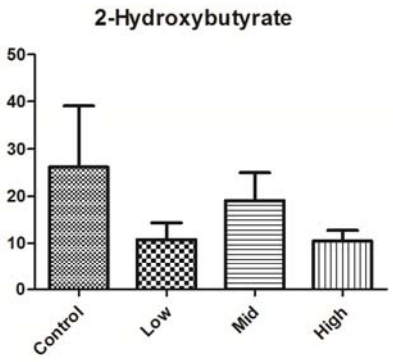
Mid
High
Groups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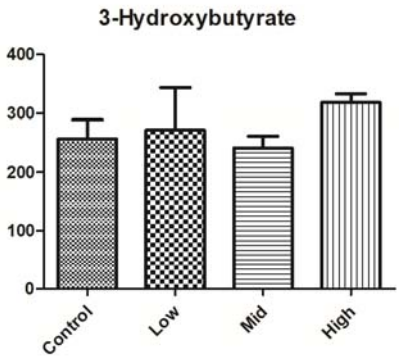
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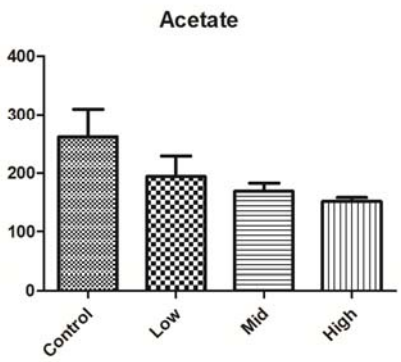
Acetate
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200
100
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Control
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Mid
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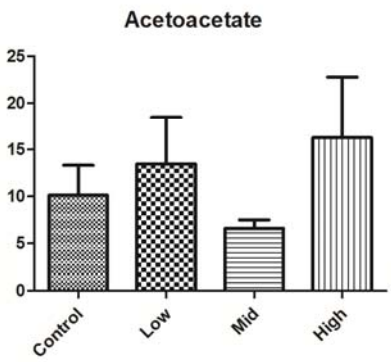
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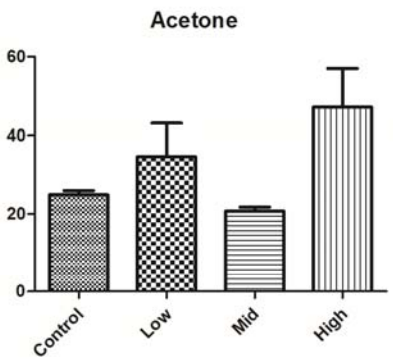
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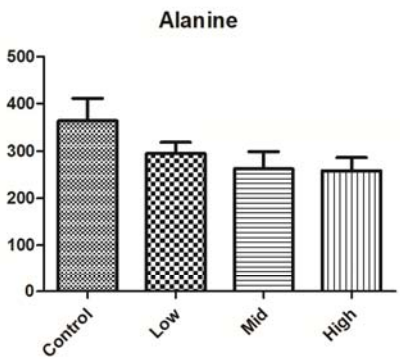
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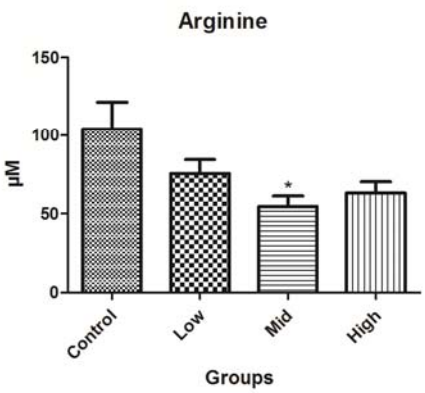
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µM
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Mid
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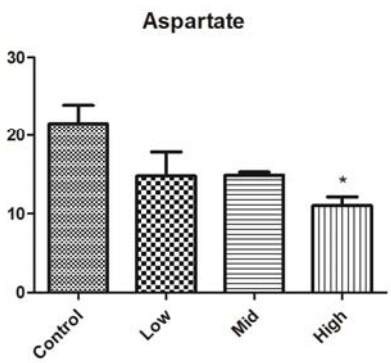
Groups


Aspartate
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10
0
*
Control
Low
Mid
High

Publications:

Paper I: LC-MS analysis of the plasma metabolome – a novel sample preparation strategy

Journal of Chromatography B

Aim: Increasing metabolite information from a rat plasma sample by solid phase extraction resulting in three sub-fractionations of the plasma metabolites.

Results: Sub-fractionation increased the chromatographic separation. Furthermore, using two different solvent systems for non-polar and polar compounds resulted in increased separation and ensured that the phospholipids did not precipitate on the analytical column. The fractionation approach was compared with a protein precipitation approach; the sub-fractionation approach resulted in 4234 compared to 1792 from the protein precipitation approach. Evaluation of the sub-fractioning approach was performed on low dose exposure, where difference was found between animals exposed to a low dose of chemicals and control.

Conclusion: The new method revealed 2.5 times more molecular features than the regular protein precipitation approach. The new method made it possible to detect differences even at low dose exposure on the metabolome.

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LC–MS analysis of the plasma metabolome—A novel sample preparation strategy

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ABSTRACT

Blood plasma is a well-known body fluid often analyzed in studies on the effects of toxic compounds as physiological or chemical induced changes in the mammalian body are reflected in the plasma metabolome. Sample preparation prior to LC–MS based analysis of the plasma metabolome is a challenge as plasma contains compounds with very different properties. Besides, proteins, which usually are precipitated with organic solvent, phospholipids, are known to cause ion suppression in electrospray mass spectrometry.

We have compared two different sample preparation techniques prior to LC-qTOF analysis of plasma samples: the first is protein precipitation; the second is protein precipitation followed by solid phase extraction with sub-fractionation into three sub-samples: a phospholipid, a lipid and a polar sub-fraction. Molecular feature extraction of the data files from LC-qTOF analysis of the samples revealed 1792 molecular features from the protein precipitation procedure. The protein precipitation followed by solid phase extraction procedure with three sub-samples gave a total of 4234 molecular features. This suggests that sub-sampling into polar, lipid and phospholipid fractions enables extraction of more metabolomic information as compared to protein precipitation alone. Chromatography showed good separation of the metabolites with little retention time drift (<1 s) and a mass accuracy below 3 ppm was observed. The performance of the method was investigated using plasma samples from rats administered the environmental pollutant perfluorononanoic acid.

1. Introduction

Metabolomics is an analytical strategy based on a comprehensive analysis of ideally, all low molecular weight compounds in a specific biological compartment [1]. This strategy is highly valuable in toxicological studies. It can be used to obtain a metabolic fingerprint that enables determination of changes in response to exposure to xenobiotic chemicals [2]. Specifically, metabolomics can be used to quantitate the amount of the xenobiotic as well as its metabolites [2–4]. The latter can provide investigators with suggestions on the nature of affected biochemical pathways and thus the nature of toxic effects and mechanisms [2].

Several analytical techniques are available to determine the metabolome. Nuclear magnetic resonance (NMR) and mass spectrometry coupled to either gas chromatography (GC–MS) or liquid chromatography (LC–MS) are the most frequent used techniques. Each of these techniques has its advantages and disadvantages. NMR requires little sample preparation but is reported to have limited sensitivity [4]. GC–MS has excellent separation capacity and good sensitivity but requires derivatisation of the sample. LC–MS has good separation capacity and good sensitivity toward most compounds. However, very polar metabolites are poorly retained on conventional LC columns [3,5]. An added benefit of high resolution accurate mass spectrometry is that it improves the possibility to identify metabolites [6]. Often, the complexity of the metabolome as described below, will nearly always call for a combination of analytical techniques to obtain a comprehensive picture of the metabolome [7].

Blood perfuse all organs in the body and its metabolome can provide comprehensive information reflecting the status of the whole bodies' physiology. While the metabolome can readily be measured in urine, representing a collection of metabolites eliminated by the organism, analysis of the plasma metabolome, which better reflects the physiology status of the organism, requires

Abbreviations: MG, monoacylglycerol; PLS-DA, partial least square discriminant analysis; PC, phosphotidylcholine; PFNA, perfluorononanoic acid.

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1570-0232/

a more laborious analytical procedure. Plasma contains a complex mixture of compounds ranging from very hydrophilic to very hydrophobic with a concentration range from picomolar to millimolar [7–10]. This complexity challenges the analytical procedures [11]. In particular, the co-occurrence of peptides/proteins together with phospholipids in plasma complicates the analysis of metabolites. Before a chromatographic analysis of plasma constituents proteins are usually removed by solvent precipitation with methanol, acetonitrile, or perchloric acid [12,13]. It has been shown that three volumes of acetonitrile per plasma volume is the most efficient protein precipitating agent [13]. After protein precipitation, the remaining compounds mainly comprise low molecular weight compounds including e.g. amino acids, organic acids, hormones, urea and uric acids as minor constituents, together with phospholipids and tri-, di- and mono-glycerides as major constituents [7]. Phospholipids do not only interfere with a good chromatographic separation, they also ionize very efficiently in electrospray ionization mass spectrometry, and hence may suppress signals from other analytes [14,15]. To obtain a comprehensive metabolic picture it is an advantage to remove phospholipids before a generic LC–MS analysis. For the present investigation we suggest that chromatographic performance will be improved by a fractionation of the remaining polar and non-polar substances in two sub-fractions.

The objective of the present investigation was to develop a 'fast and easy method' that includes a fractionation of the plasma prior to liquid chromatography–mass spectrometry (LC–MS) analysis in order to obtain good chromatographic performances for a wide range of different compounds. The fractionation method is based on Tulipani et al. [14] but developed further, to obtain valid data for phospholipids, non-polar and polar compounds in plasma. The efficacy of the method for toxicological investigations was tested by use of samples from rats administered the environmental pollutant perfluorononanoic acid, currently observed in increased concentrations in human plasma.

2. Materials and methods

2.1. Chemicals

All aqueous solutions were prepared using ultrapure water obtained from a Millipore Milli-Q Gradient A10 system (Millipore, Bedford, MA). HPLC MS grade methanol, isopropanol, 25% ammonium hydroxide, formic acid, Bergamottin (98%), glabrindin (98%), bisphenol A (99.5%), butyl paraben (99%) and 4-methylbenzylidene camphor (98%) were obtained from Sigma–Aldrich (St. Louis, MO). LiChroSolv LC–MS grade acetonitrile was obtained from Merck (Darmstadt, Germany). Dibutylphtalate (96%), bis(2-ethylhexyl)phthalate (99%), octyl methoxycinnamate (98%), dichlorodiphenyldichloroethylene (99%), epoxyconazole (99%), linuron (99.7%), prochloraz (98%) and vinclozolin (99.5%) were purchased from VWR, Bie & Berntsen, Herlev, Denmark.

2.2. Plasma extraction

100 μl plasma sample (stored at −80 °C prior to analysis) was added to an Eppendorpf vial containing 300 μl ice cold (−20 °C) acetonitrile with 2% formic acid. The mixture was incubated at −20 °C for 20 min in order to facilitate protein precipitation. After a centrifugation at 10,000 × *g* for 7 min at 4 °C (Ole Dich Instrument makers, Denmark), the supernatant was either transferred to an HPLC vial (protein precipitation only procedure) and analyzed by LC–MS or transferred to an SPE cartridge (30 mg Hybrid SPE Phospholipid column Supelco, Sigma–Aldrich, St. Louis, MO) conditioned with acetonitrile in order to sub-fractionate the sample prior to LC–MS.

The SPE column was first eluted by the addition of 300 μl of 1% formic acid in acetonitrile, and the eluate was collected in a vial and evaporated to dryness at 50 °C using a gentle stream of nitrogen. The dry residue was then extracted by adding 200 μl of heptane. The heptane fraction was then transferred to a clean vial. The residue was then extracted by adding 200 μl of methanol (polar fraction). The heptane fraction was evaporated at 50 °C using a gentle stream of nitrogen. The dry residues of the heptane fraction was dissolved in 200 μl of 50:50 (acetonitrile:isopropanol) (lipid fraction).

The hybrid SPE column was eluted by 300 μl of 10% ammonium hydroxide in methanol, in order to elute the phospholipids. The phospholipid fraction was diluted with 200 μl Milli-Q water and 500 μl of methanol.

The three fractions obtained from each plasma sample were: (1) a phospholipid fraction, (2) a lipid fraction and (3) a polar fraction. The phospholipid fraction and the lipid fraction were analyzed by a hydrophobic LC procedure and the polar fraction was analyzed by a hydrophilic LC procedure (described in Section 2.4). The fractionation lasted 8 h for 82 samples corresponding to 6 min per sample. The fractions were stored at −20 °C between the analyses. No degradation was observed by re-analysis of the sample after two weeks storage at −20 °C.

2.3. Animal study

Male Wistar rats, six weeks of age were purchased and housed as previously described [16]. Animals were divided into eight groups with 8–10 animals in each group. The animals were administered the following substances/mixtures by oral gavage:

(1) Vehicle (corn oil in a volume of 1 mL/100 g of body weight (bw)); (2) perfluorononanoic acid (PFNA) at doses of 12.5 μg/kg bw/day; (3) 12.5 μg/kg bw/day PFNA and a mixture of 14 chemicals (Mix) (the content of the mixture is described below); and (4) Mix alone.

The Mix consisted of: Bergamottin 200 μg/kg bw/day, glabridin 300 μg/kg bw/day, bisphenol A 10 μg/kg bw/day, butyl paraben 520 μg/kg bw/day, dibutylphtalate (DBP) 60 μg/kg bw/day, bis(2-ethylhexyl)phthalate (DEHP) 90 μg/kg bw/day, 4-methylbenzylidene camphor (4-MBC) 380 μg/kg bw/day, octyl methoxycinnamate (OMC), 680 μg/kg bw/day dichlorodiphenyldichloroethylene (DDE), 6 μg/kg bw/day, epoxiconazole 50 μg/kg bw/day, linuron 4 μg/kg bw/day, prochloraz 60 μg/kg bw/day, procymidone 90 μg/kg bw/day and vinclozolin 50 μg/kg bw/day. The total dose of the mixture was 2500 μg/kg bw/day.

After a treatment period of 14 days, the animals were anaesthetized in CO_2/O_2, euthanized by decapitation and a blood sample was collected in heparinized plastic tubes from the neck wound. Plasma was isolated as the supernatant following centrifugation at 1000 × *g*, 4 °C for 10 min.

2.4. LC-qTOF MS

Liquid chromatography was conducted on a Dionex Ultimate 3000 RS (Thermo Scientific, CA) with an Ascentis Express C8 (100 mm × 2.11 mm, 2.7 μm) column (Supleco, St. Louis, MO). Two other HPLC columns were tested and found not to be suitable for the purpose: a Kinetex PFP column, 100 mm × 2.1 mm, 2.6 μm (Phenomenex, Torance, CA) and an Ascentis Express cyano propyl column 100 mm × 2.1 mm, 2.7 μm (Supleco, St. Louis, MO). The solvent system consisted of (A) 5 mM ammonium hydroxide + 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Two different gradient systems were used:

The hydrophobic LC procedure: the solvent composition was isocratic 70% B for 1 min, 75% B at 3 min, 100% B at 8 min and isocratic 100% B for 2 min, followed by re-equilibration at 70% for additional

2 min. Column temperature was 50 °C. Backpressure of the column at 75% acetonitrile was 161 bar.

The hydrophilic LC procedure: the solvent composition was 0% B for 1 min, 5% B at 3 min followed by 100% B at 10 min, then isocratic at 100% B for 2 min followed by re-equilibration at 0% B for 2 min. Column temperature was 40 °C. The backpressure of the column was at the highest (15% acetonitrile) 190 bar.

The flow was 0.3 ml/min and the injection volume for the hydrophilic LC procedure and the hydrophobic LC procedure was 3 and 1 μl, respectively. The LC system was connected to a Bruker Daltonics, maXis qTOF mass spectrometer equipped with an electrospray ion source (Bruker Daltonics, Bremen, Germany). The ion source settings were as follows: nitrogen was used as nebulizer, drying and collision gas, the nebulizer pressure was two bars, the drying gas flow was 10 l/min, the dry gas temperature was 200 °C, the capillary voltage was 4500 V. The scan range was set to be from 50 to 1100 *m*/*z* in both positive and negative ionization modes, with an acquisition rate of 2 Hz. Sodium formate dissolved in 50% 2-propanol was introduced into the ion source in a 0.2–0.4 min time segment and used for internal calibration of the data files. Hexakis(1H,1H,2H-perfluoroethoxy)phosphazene was used as lock mass calibrant. Auto MS/MS was performed on one sample from each group and on reference samples, if available, in order to facilitate the identification process.

2.5. Chemometric analysis

Data was analyzed with profile analysis 2.1 (Bruker Daltonics, Bremen, Germany) using the Find Molecular Features (FMF) algorithm, including time alignment, quantile normalization and a pareto scaling model. Data were subsequently analyzed by partial least square discriminant analysis (PLS-DA), which is a method used for interpretation and visualization of profiling data. PLS-DA is a supervised method and describes the highest co-variation between the metabolite data and the response variable [17].

2.6. Identification of compounds

Identification of the compounds, differing significantly between groups, was conducted by accurate mass search in the human metabolome database (www.hmdb.ca), the Metlin database (www.metlin.scripps.edu) and in the Lipidmaps database (www.lipidmap.org). Identification was based on comparison of data on retention times, MS and MS/MS spectra with data obtained from reference compounds or based on comparison of data on MS and MS/MS spectra with similar data from the human metabolome database and Lipidmaps.

3. Results

3.1. Method development

Plasma samples were analyzed by LC–MS after protein precipitation (data not shown) or fractionated into a polar, a non-polar and a phospholipid fraction using a hybrid SPE column followed by LC–MS analysis of each sub-fraction. Fig. 1 shows the flow of the sub-fractionation approach giving three sub-fractions: phospholipid, lipid and polar (Fig. 1).

Three different HPLC columns were tested for separation efficiency of the three sub-fractions of the plasma samples, a Kinetex PFP column (Phenomenex, Torance, CA), an Ascentis Express cyanopropyl column (Supleco, St. Louis, MO) and an Ascentis Express C8 column (Supelco, St. Louis, MO). Severe bleeding was observed from the PFP column and further analyses on this column was not attempted (data not shown); the cyanopropyl column gave a satisfactory chromatography and separation of the lipid and

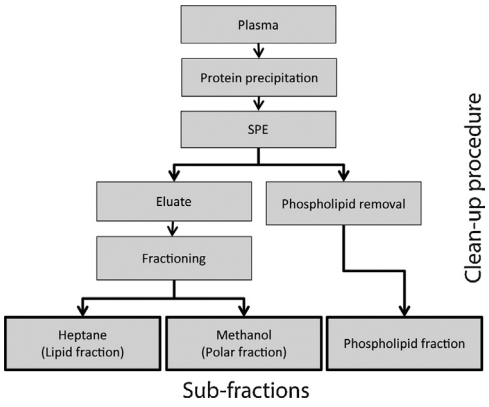

Fig. 1. Flow diagram of the SPE method. Proteins were precipitated by adding 300 μl acetonitrile with 1% formic acid followed by SPE clean-up of the supernatant in three sub-fractions.

polar fractions, but performed poorly regarding separation of phospholipids (data not shown). The C8 column resulted in satisfactory chromatographic resolution of all three different classes of compounds (Fig. 2). Examination of the data files using the molecular feature extraction algorithm resulted in 1792 molecular features from the LC–MS analysis of the protein precipitated sample. Molecular feature extraction on the data files from LC–MS analyses of polar, lipid and phospholipid sub-samples resulted in 3172, 804 and 258 molecular features, respectively. A limited number of the compounds were observed in both the lipid and the polar fraction. The sub-fractionation thus resulted in the extraction of totally 4234 molecular features compared to 1792 features using only protein precipitation.

The chromatogram (Fig. 2a) shows the separation of the phospholipid fraction which eluted in two groups of primarily phosphocholine (PC) compounds. The first group of peaks eluting between 60 and 120 s were low molecular weight lysophosphotidylcholine containing one fatty acid chain e.g. PC (20:0) and PC (18:0) while the compounds eluting between 340 and 470 s were high molecular weight compounds containing two fatty acid chains e.g. phosphotidylcholine (20:4/16:0). The phospholipid fraction molecular features were identified by an accurate mass search in HMDB, followed by MS/MS examination of the chromatographic peak. Each of the phosphotidylcholine compounds had a characteristic 184.073 *m*/*z* fragment.

The chromatogram of the lipid fraction (Fig. 2b) contained the neutral lipids such as tri, di- and monoacylglycerols. The small peaks in the beginning of the chromatogram were relatively polar lipids, while the sharp peaks around 400–500 s were one chained lipids e.g. monoacylglycerols (MGs). The broader peaks between 500 and 700 s were neutral lipids with two and three chains e.g. di- and triglycerides. The lipids were identified by accurate mass search and comparison of MSMS pattern with the in silico MS/MS pattern from Lipidmaps.

The chromatogram of the polar fraction (Fig. 2c) contained small organic compounds, hormones such as cortisone and hydroxyl cortisone, but also monoacylglycerol (16:0) and monoacylglycerol (18:0). The peaks eluting around 300–400 s were low molecular weight compounds with masses ranging from 300 to 500 *m*/*z* e.g. corticosterone, while MG (18:0) was present at a retention time of 630 s. MG (16:0) and MG (18:0) were identified based on exact mass and by comparing MS/MS data with data from a standard. The presence of a double bond in the acyl chain of the fatty acid reduced the retention time by approximately 30 s as compared to the retention time of the saturated monoacylglycerols.

The repeatability of the LC–MS method was investigated by multiple injections of a single sample on different time points on

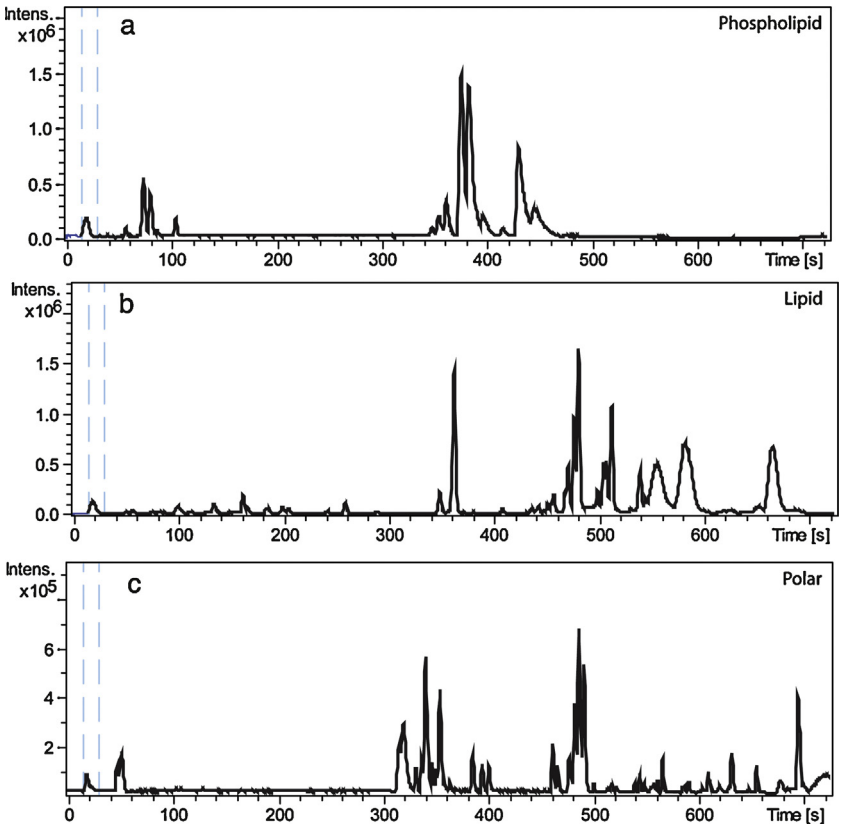

Fig. 2. Three base peak chromatograms from a sub-fractionated control plasma sample: (a) phospholipid fraction, (b) lipid fraction and (c) polar fraction. The (a) and (b) were analyzed by the hydrophobic gradient system (Section 2) while (c) was analyzed by the hydrophilic gradient system (Section 2).

Table 1
The differences in retention times, in peak intensity and the in mass deviation of the three sub-fractions after analysis of nine injections of a single sample on two different days ($n = 18$). The values are based on three peaks eluting to different time in the chromatogram (data shown in supplementary materials).

Fraction	ΔTime (s)	ΔIntensity (%)	Δm/z (ppm)
Phospholipids	<1	2.9–10.4	1.4–1.6
Lipid	<1	6.4–11.1	3.1–3.4
Polar	<1	3.4–8.3	1.0–2.4

a single day as well as on different days. These chromatography data showed a high degree of reproducibility (data not shown). The retention time stability was within 1 s for peaks in all three fractions and the mass accuracy and peak intensity only exhibited minor variations as displayed in Table 1. To evaluate the repeatability of the SPE method the intensity of three selected peaks from each of the sub-fractions was determined in the control animals ($n = 10$). The deviations (S.D.) were 21.1%, 33.1%, and 29.5% for the phospholipid, lipid and polar fraction respectively. These deviations will also include the variation between the animals in addition to the variation SPE clean-up method.

The repeatability of the method was further investigated by spiking of plasma samples with 12 different reference compounds, i.e. three phospholipids, three lipids and 6 polar compounds. All reference compounds were detected in the expected fractions; the three phospholipids in the phospholipid fraction, the three neutral lipids in the lipid fraction and the six polar compounds in the polar fraction. The capacity of the Hybrid SPE cartridges was exceeded when the sample was spiked with high concentration of phospholipids (0.3 mg/ml) in that case the sample size was reduced.

3.2. Application of the method on an animal study

To test the performance of our analytical procedure, we analyzed plasma samples from an animal study designed to investigate the effect on mammalian physiology of perfluorononanoic acid (PFNA) administered in combination with a mix of 14 chemicals (Mix) [18].

Partial least squares discriminant analysis (PLS-DA) of LC–MS data from the polar fraction and the phospholipid fraction of the plasma sample is shown in Fig. 3. The two plots were used to illustrate the sensitivity of the method (Fig. 3).

The PLSDA score plot (Fig. 3a) of the polar fraction showed that animals dosed with PFNA plus mix clustered close to animals dosed with mix alone, however distanced from animals dosed with PFNA and controls suggesting that dosing with mix clearly affects the clustering results. Control animals and animals dosed with PFNA alone were located more scattered in the score plot. The score plot, Fig. 3b, of the phospholipid fraction also showed a tendency to clustering of the animals dosed with PFNA plus Mix and animals dosed with Mix alone and separated from the clustering of control animals and animals dosed PFNA alone.

4. Discussion

4.1. Solid phase extraction vs. protein precipitation

The first attempt on sample preparation of plasma samples was based on a paper by Souverain et al., using protein precipitation with an acidified organic solvent, in this case acetonitrile, followed by LC–MS [13]. Chromatographic analysis of these extracts showed poor chromatographic separation with high signals from the

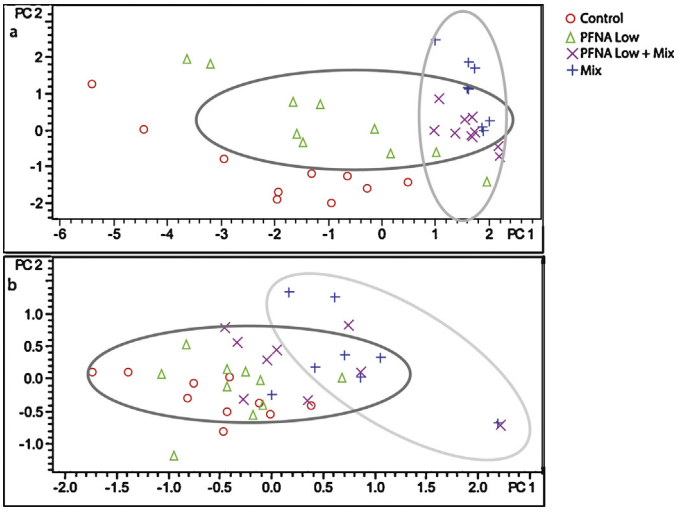

Fig. 3. PLS-DA score plots of data from the polar and the phospholipid fraction. Score plot of the polar fraction display a clustering of Mix and PFNA low + Mix distant from control and PFNA low (a). The score plot of the phospholipid fraction display a clustering of Mix and PFNA low + Mix separating from PFNA low and control (b).

phospholipids causing ion suppression in the mass spectrometer. This resulted in a loss of low intensity ions with similar retention times (data not shown). As a result of these observations, it was concluded that a fractionation of the samples was needed. Hence, a method based on Tulipani et al. [14] was implemented in which a solid phase extraction (SPE) column was used to remove the phospholipids. However, as changes in the phospholipid pattern gives important information on mammalian physiology the method was extended to include these. Therefore, the extraction/SPE procedure was adjusted. First the phospholipids were isolated. Then the eluate was chemically separated into two fractions by first extracting with heptane to isolate a lipid fraction and then extracting with methanol to isolate the polar compounds. As discussed by Tulipani et al. [14] the removal of phospholipid reduces the ion suppression in the mass spectrometry, but at the cost of a reduced number of identified molecular features. However, by eluting the phospholipids from the SPE column information from this group of compounds was retained.

Metabolic information on the phospholipids is important in physiological studies [7], as exemplified by Kim et al. [19] who showed that phospholipids were affected by hepatic steatosis. Furthermore, phospholipids are believed to have signaling roles in the plasma [20] and inside cells. Therefore, changes in phospholipid levels may provide information on specific mechanisms and effects in mammals. From this perspective it was important to have these compounds included in the analysis.

The total number of molecular features extracted from the chromatograms after the sub-fractionation approach were 4234. Using only protein precipitation as described in Souverain et al. 1792 molecular features were extracted from the chromatogram [13]. The 4234 molecular feature obtained after sub-fractionation will include some compounds twice as these are equilibrium distributed between the heptane and the methanol extraction solvent. The fractionation approach increases the amount of information obtained on the plasma metabolome. Furthermore, the separation in three fractions yielded more chromatographic peaks and increased the sensitivity as the ion suppression was reduced. It was found that a C8 column was the best compromise to give a good chromatographic separation of all three sub-fractions: polar, lipid and phospholipid. By using a single analytical column all three sub-fractions could be analyzed in one sequence saving time and reducing cost of the analyses.

Although to achieve good chromatographic separation two gradient systems have to be used. The lipid and phospholipid fraction required a gradient starting at 75% organic solvent to prevent precipitation on the column as these compounds are highly non-polar. On the other hand, the polar fraction contains more water soluble compounds; therefore we started the gradient as low as 0% organic. In spite of the gradient starting at 0% organic, the chromatograms showed only few early eluting peaks. This was most likely due to poor ionization of the polar compounds in the electrospray ionization (ESI) interphase. Generally, it would be preferable to analyze all the metabolites using a single chromatographic system. However, this is not possible with such a wide range of metabolites [21]. The repeatability was evaluated using data from the control group and the deviations (S.D.) were determined to be 21.1%, 33.1%, and 29.5% for the phospholipid, lipid and polar fraction respectively.

It was found that the HPLC system was reproducible for all three sub-fractions with a retention time deviation below 1 s. Though little retention time change was observed it may still be possible to improve the peak shape for the non-polar compounds. Choi et al. suggested that addition of 50% isopropanol to the organic solvent of the HPLC system yielded sharper peaks, at least for the phospholipids [22]. However, this was not implemented in the current study. The mass deviation was found to be between 1 and 3.4 ppm which is more than suitable for identification of the compounds present in the plasma metabolome.

4.2. Application of the method on plasma samples from an animal study

The method was established to provide maximum information on the plasma metabolome from animal experiments designed to study the effects of dosing with chemicals. The HPLC analyses showed good chromatographic separation for each of the three sub-fractions opening the possibility to identify critical components based on the exact mass. Using a PLS-DA analysis it was possible to detect differences in the metabolome even a low exposure levels, as shown by the clustering in the score plot, Fig. 3. The PLS-DA plot of both the polar and phospholipid fraction showed a clustering of PFNA Mix and Mix dosed animals, distanced from PFNA and control animals. This suggests that the change of the metabolome is primarily driven by dosing Mix rather than PFNA. The Mix was administered to the animals at a dose comparable to high end human exposure revealing the applicability of the analytical method.

5. Conclusion

We developed a method to improve sub-fractionate plasma samples into a phospholipid, a lipid and a polar sub-samples. These were analyzed by two different LC gradient systems combined with high resolution mass spectrometry. This method was more time consuming than a protein precipitation approach, but the improved chromatographic separation and reduced ion suppression compared to protein precipitation providing more data from each plasma sample. Furthermore, the number of molecular features detected increased from 1972 to 4234. The sub-fractionation divided the samples into 3 different chemical classes and knowledge of the chemical identity of the metabolites polar, lipids and phospholipids is an advantage in the identification of the metabolites. The method was demonstrated to exhibit repeatable chromatographic results for each of the three fractions with a time shift of less than 1 s and a mass deviation below 3.4 ppm. Furthermore, the injection on two separate days showed similar peak intensity and chromatography, this shows that separation and purification by the SPE column is reproducible. The

usefulness of the method was demonstrated by the detection of overall differences in the metabolome of animals administered a dose comparable with human exposure of the endocrine disruptor perfluorononanoic acid.

Acknowledgement

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.11.033.

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Paper II: Perfluorononanoic acid in combination with 14 chemicals exerts low-dose mixture effects in rats

Archives of Toxicology

Aim: A toxicological study aimed at investigating whether adverse mixture effects do not occur at doses approaching high-end human exposure levels.

Result: Indication of a toxicokinetic interaction was found, as simultaneous exposure to PFNA and the Mix caused a 2.8-fold increase in plasma PFNA concentrations at Low PFNA. Corticosterone, the stress hormone of rats was increased with PFNA alone; while androstenedione, testosterone and dihydrotestosterone were significantly increased in by 0.0125 mg/kg bw/day PFNA with mix and not low PFNA alone.

Conclusion: PFNA affects the steroid hormone synthesis in rats. Corticosterone, was affected by PFNA while for androstenedione, testosterone and dihydrotestosterone the induced effect was caused by the combination with the mixture.

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ORGAN TOXICITY AND MECHANISMS

Perfluorononanoic acid in combination with 14 chemicals exerts low-dose mixture effects in rats

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Abstract Humans are simultaneously exposed to several chemicals that act jointly to induce mixture effects. At doses close to or higher than no-observed adverse effect levels, chemicals usually act additively in experimental studies. However, we are lacking knowledge on the importance of exposure to complex real-world mixtures at more relevant human exposure levels. We hypothesised that adverse mixture effects occur at doses approaching high-end human exposure levels. A mixture (Mix) of 14 chemicals at a combined dose of 2.5 mg/kg bw/day was tested in combination with perfluorononanoic acid (PFNA) at doses of 0.0125 (Low PFNA), 0.25 (Mid PFNA) and 5 (High PFNA) mg/kg bw/day by oral administration for 14 days in juvenile male rats. Indication of a toxicokinetic interaction was found, as simultaneous exposure to PFNA and the Mix caused a 2.8-fold increase in plasma PFNA concentrations at Low PFNA. An increase in testosterone and dihydrotestosterone plasma concentrations was observed for Low PFNA + Mix. This effect was considered non-monotonic, as higher doses did not cause this effect. Reduced LH plasma concentrations together with increased androgen concentrations indicate a disturbed pituitary-testis axis caused by the 15-chemical mixture. Low PFNA by itself increased the corticosterone plasma concentration, an effect which was normalised after simultaneous exposure to Mix. This combined with affected ACTH plasma concentrations and down-regulation of 11β HSD mRNA in livers indicates a disturbed pituitary-adrenal axis. In conclusion, our data suggest that mixtures of environmental chemicals at doses approaching high-end human exposure levels can cause a hormonal imbalance and disturb steroid hormones and their regulation. These effects may be non-monotonic and were observed at low doses. Whether this reflects a more general phenomenon that should be taken into consideration when predicting human mixture effects or represents a rarer phenomenon remains to be shown.

Keywords Mixture toxicology · Steroidogenesis · Testosterone · Corticosterone · Pituitary hormones · Perfluorononanoic acid (PFNA)

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Introduction

Humans are concomitantly exposed to several chemicals that can exert mixture effects. At doses close to no-observed adverse effect levels or higher, chemicals usually act additively in experimental studies (Kortenkamp 2014). However, we are lacking knowledge on the importance of exposure to complex real-world mixtures at more relevant human exposure levels. Only few in vivo studies performed with low-human relevant exposure levels have been reported. Wade et al. investigated a mixture containing 18 persistent contaminants at a dose of 1x the estimated safe level. This dose was based on the minimum risk levels (MRLs) or the tolerable daily intakes and was dosed orally to male rats for 70 days. At this dose, increased natural killer cell lytic activity was observed (Wade et al. 2002). Crofton et al. investigated whether deviations from additivity could be detected at low doses. They studied

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thyroid disruptors in doses ranging from environmental background to 100x environmental background in rats for effects on serum total thyroxine. No deviation from additivity was found at the lowest doses (<0.2 mg/kg bw/day), but at higher doses (>0.67 mg/kg bw/day), synergy was found (Crofton et al. 2005). Stanko et al. found an increase in prostate inflammation with 0.09 mg/kg bw of an atrazine metabolite mixture following prenatal exposure of male rats (Stanko et al. 2010). Merhi et al. exposed mice to a mixture of six pesticides at levels derived from the human acceptable daily intake levels. Changes in blood cell counts were observed (Merhi et al. 2010). Demur et al. (2013) found dietary exposure to a low atrazine, endosulfan and chlorpyrifos mixture dose (25, 30 and 50 μg/kg food corresponding to approximately 4, 5, and 8 μg/bw/day) to decrease the red blood cell count and haemoglobin levels.

To further address exposure to human relevant mixtures of chemicals, we designed an experiment to test whether chemicals in food approaching high-end human exposure cause adverse mixture effects. We hypothesised that effects could occur via toxicokinetic metabolism effects comparable to those described for food–drug interactions, e.g., as has been described for grapefruit juice and Ca^{2+} channel blockers (Bailey et al. 1989). For our investigation, we used steroid hormone metabolism as an effect measure. Steroid hormones are involved in multiple important male developmental processes and thus alterations in its plasma concentration may potentially be accompanied by adverse effects. We designed an experiment in which a single chemical known to affect the steroid hormone testosterone on its own was tested at increasing doses in the presence or absence of a fixed dose of a 'background' chemical mixture (Mix). On basis of its human toxicological importance and on basis of its ability to increase testosterone at a dose of 1 mg/kg bw/day to rats (Feng et al. 2009), we selected the fluorosurfactant perfluorononanoic acid (PFNA) as the variably dosed chemical in this investigation. We exposed the rats to a fixed background dose of 14 chemicals—12 environmental chemicals representing typical endocrine disrupting chemicals and two food ingredients. For the design of this Mix, we took advantage of the fact that testosterone is metabolized in the liver by cytochrome P450 3A4 (CYP3A4 homologous to CYP3A23/3A1 in rat) and CYP2C9 (homologous to CYP2C11 in rat) (Cheng and Schenkman 1983; Guengerich 1999; Kenworthy et al. 1999; Martignoni et al. 2006). CYP3A4 and CYP2C9 are inhibited by the grapefruit constituent bergamottin and the liquorice constituent glabridin (Bailey et al. 2003; Foti and Wahlstrom 2008; Kent et al. 2002; Lim et al. 2005; Tassaneeyakul et al. 2000; Uesawa and Mohri 2006; Wen et al. 2002). These substances were included in the Mix along with twelve food contaminants (described in Table 2), each reported to exert endocrine disrupting effects (Christiansen et al. 2012). The doses of these twelve contaminants were selected to reflect a high-end exposure level to the European human population. Thus, to test whether chemicals in food at human relevant doses caused adverse mixture effects, we administered PFNA at doses 0.0125 (Low PFNA), 0.25 (Mid PFNA) and 5 (High PFNA) mg/kg bw/day in the presence or absence of Mix at a total dose of 2.5 mg/kg bw/day (Table 2) to male rats for 14 days. Steroid hormone plasma concentrations, pituitary hormone levels as well as classical toxicological parameters and mechanistic markers in testis, liver, adipose tissue and kidney were investigated.

Methods

Dose selection and chemicals

We aimed at selecting a PFNA dose to cover human internal exposure of the combined exposure to perfluorinated compounds such as PFOA, PFOS and PFNA. Lau et al. (2007) gathered human exposure data from a large number of studies. The PFOS mean plasma concentrations in single studies were 10–73 ng/mL (see Table 1 for an overview of the data in table format), for PFOA the values were 2.1–354 ng/mL (second highest value was 41 ng/mL), and for PFNA 0.8–2.2 ng/mL. This gives a possibility of a combined human exposure to these three perfluorinated compounds of 13–429 ng/mL (or 13–117 ng/mL if only the second highest PFOA value is used) (Lau et al. 2007). For comparison, the high-end exposure (95th percentile) in the American population as determined by the National Health and Nutrition Examination Survey (NHANES) is 67.6 ng/mL for PFNA, PFOA and PFOS combined (CDC 2009). The PFNA dose employed in the present investigation was chosen on basis of a study by Tatum Gibbs et al. (Tatum-Gibbs et al. 2011) in which an oral dose of 1 mg/kg bw to rats gave a serum concentration of approximately 10,000 ng/mL. From that we chose a dose of 0.0125 mg/kg bw/day for a 14-day investigation (Abbreviated: Low PFNA), predicted giving rise to a PFNA plasma concentration of 125 ng/mL. For a medium- and high-dose level, we included 0.25 (Mid PFNA) and 5 (High PFNA) mg/kg bw/day.

We aimed to administer bergamottin in an amount corresponding to the content of one grapefruit. A grapefruit contains 25 μM bergamottin (Bailey et al. 2003) corresponding to 1,700 μg bergamottin per grapefruit. Adjusting according to body surface area as described (Reagan-Shaw et al. 2008), a rat dose corresponding to a human intake of 100 mL grapefruit juice was calculated to be 0.2 mg/kg bw/day. For glabridin, an amount corresponding to an intake of 100-g liquorice candy per day was determined in the following way. The extraction of glycyrrhizic acid and glabridin from Chinese (raw) liquorice gives 2.39 mg/g

Table 1 Comparison of reported human perfluorinated compound levels with measured levels in rats

	Reported human exposure levels to PFCs (ng/mL)		
	NHANES (geometric means) (CDC 2009)	NHANES 95th percentile (CDC 2009)	Mean value ranges from literature reviewed in Lau et al. (2007)
PFNA in serum	1.0	3.2	0.8–2.2 ($n = 6$)
PFOA in serum	4.0	9.8	2.1–354 ($n = 9$)
PFOS in serum	20.7	54.6	10–73.2 ($n = 11$)
Total PFC in serum	25.7	67.6	32.8–429

	Measured rat exposure levels (ng/mL)	Comparison human versus rat
Measured PFNA plasma conc. in rats at low dose	396	0.9- to 15-fold human exposure to total PFC Sixfold human 95th percentile NHANES exposure
Measured PFNA plasma conc. in rats at mid dose	29,950	70- to 1,200-fold human exposure to total PFC 440-fold human 95th percentile NHANES exposure

NHANES National Health and Nutrition Examination Survey, *PFC* perfluorinated compound

glycyrrhizic acid and 0.92 mg/g glabridin (Tian et al. 2008). This taken together with a reported 150 mg glycyrrhizic acid in 100 g of sweet liquorice (candy) (Sigurjonsdottir et al. 2001), suggests an amount of 58 mg glabridin in 100 g of sweet liquorice, suggesting that a 70-kg person takes in 0.8 mg/kg bw/day of glabridin when ingesting 100 g of sweet liquorice. This suggests—by a body surface area conversion (Reagan-Shaw et al. 2008)—that we should give a dose of 4 mg/kg bw/day to rats. However, Furhman et al. previously reported that a glabridin dose to rats corresponding to the selected human intake was 0.3 mg/kg bw/day (Fuhrman et al. 1997) and to take a conservative approach we settled for this dose. Regarding the remaining chemicals in the mixture, a previously designed mixture based on exposure levels of the European human population was used (Christiansen et al. 2012; Hadrup et al. 2013). The dose reported to be a realistic 'high-end human exposure level' (Christiansen et al. 2012) was 0.32 mg/kg bw/day. By conversion to rat dose via body surface area, this yields 2 mg/kg bw/day. The chemicals and their ratio in Mix are described in Table 2. Chemicals were purchased as follows: Bergamottin, glabridin, bisphenol A, butyl paraben and 4-methylbenzylidene camphor (4-MBC) were purchased from Sigma-Aldrich, Brøndby, Denmark. Dibutylphthalate (DBP), bis(2-ethylhexyl)phthalate (DEHP), 4-MBC, 2-ethylhexyl-4-methoxycinnamate (OMC), dichlorodiphenyldichloroethylene (DDE), epoxiconazole, linuron, prochloraz, procymidone and vinclozolin were purchased from VWR, Bie & Berntsen, Herlev, Denmark.

Animals and pathology

Male Wistar Hannover Galas rats, 6 weeks of age with specific pathogen-free health status, were obtained from Taconic M&B (Lille Skensved, Denmark) and allowed to acclimatise for 1 week. The animals were housed two per cage (Macrolon, Buguggiate, Italy) with light on from 7 a.m. to 7 p.m. Room temperature and relative humidity were 22 ± 1 °C and 55 ± 5 %, respectively. Rats were given ad libitum access to citric acid acidified tap water and standard diet (prod. no. 1324 Altromin, Brogården, Gentofte, Denmark). The animals were administered test substances once a day orally by gavage for 14 days with corn oil (VWR—Bie & Berntsen, Herlev, Denmark) as vehicle. The dosing volume was 1 mL/100 g of body weight (bw). In total, 70 male rats were randomly placed into eight groups, i.e., vehicle control ($n = 10$), PFNA 0.0125 mg/kg/day (Low PFNA) ($n = 10$), PFNA 0.25 mg/kg/day (Mid PFNA) (n = 8), PFNA 5 mg/kg/day (High PFNA) ($n = 8$), Mix + PFNA 0.0125 mg/kg/day (Low PFNA + Mix) ($n = 10$), Mix + PFNA 0.25 mg/kg/day (Mid PFNA + Mix) ($n = 8$), Mix + PFNA 5 mg/kg/day (High PFNA + Mix) ($n = 8$), Mix ($n = 8$). Animals were evaluated clinically and subjected to necropsy in four different sets each starting 1 day after the prior. Each set comprised animals of each dosing group. For the euthanisation, the animals were anaesthetised in CO_2/O_2 and decapitated. Neck blood was collected in heparinised tubes, and plasma was isolated by centrifugation at 1,000×*g*, 4 °C for 10 min. Plasma was stored at −80 °C. To avoid bias, e.g., due to stress in animals, the sectioning of animals were randomised according to groups. Animals were given the last dose in the time span of 1 h and 15 min to 1 h and 45 min before euthanisation. Body weight and organ weights (liver, kidney, testes) were recorded, and livers were fixed and processed for histopathological examination as previously described (Hadrup et al. 2012). Liver haematoxylin and eosin stained sections were evaluated by a pathologist blinded to treatment groups. Changes were described qualitatively, and in addition, selected parameters were scored in the following manner: Cell borders (score

Table 2 Fourteen chemicals in fixed ratio mixture

CAS registry number	Chemical name	Source/use	Ratio in mixture (weight)	Rat dose (mg/kg bw/day)
7380-40-7	Bergamottin	Grapefruit constituent	0.08	0.2
59870-68-7	Glabridin	Liquorice constituent	0.12	0.3
80-05-7	Bisphenol A	Plastic additive	0.004	0.01
94-26-8	Butyl paraben	Preservative	0.21	0.52
84-74-2	Dibutylphthalate (DBP)	Plasticiser	0.02	0.06
117-81-7	Bis(2-ethylhexyl)phthalate (DEHP)	Plasticiser	0.03	0.09
36861-47-9	4-Methylbenzylidene camphor (4-MBC)	Sun filter	0.15	0.38
5466-77-3	2-Ethylhexyl-4-methoxycinnamate (OMC)	Sun filter	0.27	0.68
72-55-9	Dichlorodiphenyldichloroethylene (p,p′-DDE)	Pesticide	0.002	0.006
133855-98-8	Epoxiconazole	Pesticide	0.02	0.05
330-55-2	Linuron	Pesticide	0.002	0.004
67747-09-5	Prochloraz	Pesticide	0.025	0.06
32809-16-8	Procymidone	Pesticide	0.035	0.09
50471-44-8	Vinclozolin	Pesticide	0.021	0.05
In total	14 Chemicals		1.0	2.5

0 = not visible, score 1 = not clear, score 2 = clear) and cell size (score 1 = small/normal, score 2 = slight increase, score 3 = marked increase).

PFNA plasma concentration measurement

For each PFNA dose, plasma concentrations were measured. To 20 µl plasma, 60 µl ice-cold acetonitrile was added. The sample was incubated at −20 °C for 20 min and centrifuged at 10,000×*g* for 7 min. The supernatant was used for analysis. A matrix-assisted standard curve was established using PFNA and a standard plasma sample (Precinom U, Roche Diagnostics, Hvidovre, Denmark). Samples from the 0.0125 mg/kg bw/day administration group were not diluted, 0.25 mg/kg bw/day samples were diluted tenfold, and the 5 mg/kg bw/day samples were diluted 100-fold. The analysis was conducted using a high-resolution maxis qTOF instrument (Bruker Daltonics, Bremen, Germany) coupled to an Agilent 1200 (Agilent Technologies, USA) with a Supelco C8 (100 × 2.0 mm, 1.7 µm) run in negative mode ionisation with a mass scan from 50 to 800 m/z. The gradient was 0 % B, 0 min—5 % B, 2 min—100 % B, 10 min—100 % B, 12 min—0 % B, 12.1 min—0 % B, 14 min. To test the repeatability of the method, the Low PFNA groups were analysed on two separate days. These measurements gave similar results. In order to investigate the metabolism of PFNA, a targeted approach was used to identify possible metabolites. The metabolites searched for were PFNA + glucuronic acid (+176 m/z value) and PFNA + glycine (+75 m/z value). The targeted approach was conducted using the Bruker Daltonics software: Target Analysis (Bruker Daltonics, Bremen, Germany).

Hormone measurements

Plasma samples were added internal standards (testosterone-d2 and methyltestosterone-d3), deproteinised with addition of acetonitrile and ultracentrifugation, and steroid hormones were extracted using a C18 end-capped solid-phase extraction cartridge (500 mg, 3 ml) (Merck, Darmstadt, Germany). Impurities were removed from the cartridge with demineralised water followed by elution of steroid hormones from the cartridge with methanol. The extract was evaporated to dryness with nitrogen and re-suspended in 40 % acetonitrile. Steroid hormones were separated, detected and quantified using the LC–MS/MS method previously described (Mortensen and Pedersen 2007). However, to accommodate more hormones, minor modifications were made. An Ascentis Express C8 column (2.1 × 100 mm, 2.7 µm) (Supelco) was added to the LC system (Agilent 1100). Steroids were measured with an injection volume of 10 µL in ESI+ mode with acetonitrile and water/0.2 % formic acid as the mobile phases (flow rate 0.25 mL/min, gradient method). The MS was a Quattro Ultima Triple Quadropole Instrument (Waters Corp., Milford, MA, USA). For quantification, external calibration standards were run before and after the samples at levels of 0.1, 0.5, 1.0, 2.0, 5.0 and 10 ng/mL (with 4.0 ng/mL of internal standards). The absolute recoveries of the hormones in plasma samples were estimated to be 42–94 %, based on the absolute recoveries of the internal standards in >30 experiments. The limit of quantification (LOQ) of each of the hormones in the plasma samples was estimated as the concentrations corresponding to six times signal-to-noise and was <100 pg/mL for testosterone (α- and β-isomer), progesterone, corticosterone and hydroxycortisol, <200

Table 3 Primer/probe sets for mRNA measurements

Gene	Prod no./sequence	Tissues tested
Aldo–keto reductase family 1 member C1 (AKR1C1)	Prod. No. Rn01487552_m1, Life Technologies Europe BV, Nærum, Denmark	Liver
Benzodiazepine receptor (BZRP)	Forward, 5′-TGG TTC CCT TGG GTC TCT ACA CT-3′	Testes
	Reverse, 5′-CAC CCC ACT GAC AAG CAT GA-3′	
	Probe: 5′-FAM-AAA GCC CAG CCC ATC T-MGB-3′	
Cytochrome P450 (CYP) 1A1	Prod. No. Rn00487218_m1, Life Technologies Europe BV, Nærum, Denmark	Liver
CYP1A2	Prod. No. Rn00561082_m1, Life Technologies Europe BV, Nærum, Denmark	Liver
CYP2B6	Prod. No. Rn00597739_m1, Life Technologies Europe BV, Nærum, Denmark	Liver
CYP2C11	Prod. No. Rn01502203_m1, Life Technologies Europe BV, Nærum, Denmark	Liver
CYP3A23/3A1	Prod. No. Rn03062228_m1, Life Technologies Europe BV, Nærum, Denmark	Testes
CYP11A	Forward 5′-ACG ACC TCC ATG ACT CTG CAA T-3′	Testes/adipose tissue
	Reverse: 5′-CTT CAG CCC GCA GCA TCT-3′	
	Probe: 5′- FAM-CCT TTA TGA AAT GGC ACA CAA CTT GAA GGT CA-TAMRA-3′	
CYP17	Forward: 5′-GCC ACG GGC GAC AGA A-3′	Testes/adipose tissue
	Reverse: 5′-CCA AGC CTT TGT TGG GAA-3′	
	Probe: 5′-FAM-CGT CAA CCA TGG GAA TAT GTC CAC CAG A-TMARA-3′	
CYP19	Forward: 5′-AGAACGGTCCGCCCTTTCT-3′	Testes/adipose tissue
	Reverse: 5′-TGGATTCCACACAGACTTCTACCA-3′	
	Probe: 5′-FAM-AGCTCTGACGGGCCCTGGTCTTATTC-TAMRA-3′	
3β-hydroxysteroid dehydrogenase (3β HSD)	Prod. No. Rn01789220_m1, Life Technologies Europe BV, Nærum, Denmark	Liver
11β HSD	Prod. No. Rn00567167_m1, Life Technologies Europe BV, Nærum, Denmark	Liver
17β HSD	Prod. No. Rn00588942_m1, Life Technologies Europe BV, Nærum, Denmark	Testes/adipose tissue
5α reductase	Prod. No. Rn00575595_m1, Life Technologies Europe BV, Nærum, Denmark	Testes/adipose tissue
18 s-ribosomal RNA	Forward:, 5′-GCC GCT AGA GGT GAA ATT CTT G-3′	Liver/testes/adipose tissue
	Reverse: 5′-GAA AAC ATT CTT GGC AAA TGC TT-3′	
	Probe: 5′-FAM-ACC GGC GCA AGA CGA ACC AGA G-TAMRA-3′	
Steroidogenic acute regulatory protein (StAR)	Forward, 5′-CCC TTG TTT GAA AAG GTC AAG TG-3′	Testes
	Reverse, 5′-TGA AAC GGG AAT GCT GTA GCT-3′	
	5′-FAM-CTG ACT CCT CTA ACT CCT GTC TGC CTA CAT GGT-TAMRA-3′	
UDP-glucuronosyltransferase 2B15 (UGT2B15)	Prod. No. Rn00755925_m1, Life Technologies Europe BV, Nærum, Denmark	Liver
UGT2B17	Prod. No. Rn01790037_g1, Life Technologies Europe BV, Nærum, Denmark	Liver

pg/mL for androstenedione and hydroxyprogesterone, and <2,000 for dihydrotestosterone and hydroxytestosterone. Pituitary hormone plasma concentration measurements were done by use of the Milliplex Map Rat Pituitary Kit (Prod no. RPT86K, Millipore Corporation, St. Charles, MO, USA). This was done according to the protocol of the manufacturer by use of a Luminex 100 apparatus (Bio-Rad, Hercules, CA, USA).

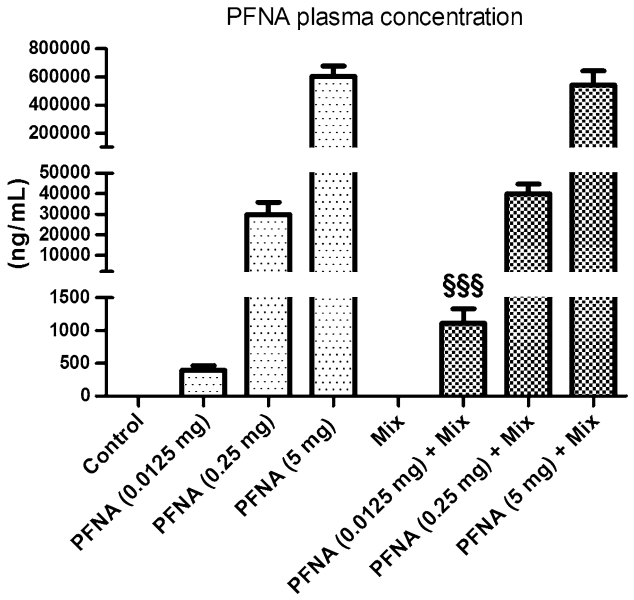


Fig. 1 Plasma concentration of PFNA in the rats. Rats were administered PFNA at 0.0125, 0.25 or 5 mg/kg bw/day for 14 days in the presence or absence of a mixture of 14 chemicals (Mix) at a total dose of 2.5 mg/kg bw/day. The PFNA plasma concentration was measured in blood from all rats at the end of the experiment using qTOF. PFNA was found in all PFNA treatment groups. At 0.0125 mg/kg bw/day (Low) PFNA, the plasma level was 396 ng/mL for Low PFNA and 1,111 ng/mL for Low PFNA + Mix. At 0.25 mg/kg bw/day, the values were 29,950 and 39,880 ng/mL for Mid PFNA and Mid PFNA + Mix, respectively. For 5 mg/kg bw/day, the values were 602,000 and 541,700 ng/mL for High PFNA and High PFNA + Mix, respectively. Data are mean plus SEM. *N* was 8 except for control and Low PFNA + Mix where *n* was 10. A *t* test was applied with $^{§§§}p < 0.001$ for Low PFNA versus Low PFNA + Mix

mRNA measurements

Organs were stored in RNAlater at −20 °C until purification by use of the RNAeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA synthesis by use of the Omniscript RT kit (Life Technologies Europe BV, Nærum, Denmark). mRNA levels were next measured by quantitative (q)PCR using specific primer pairs in combination with TaqMan probes (sequences and investigated organs are specified in Table 3). Samples, primers and probes were added TaqMan Fast Universal PCR Master Mix (Life Technologies Europe BV, Nærum, Denmark) and run on a TaqMan 7900 HT qPCR machine (Applied Biosystems, Nærum, Denmark). Quantification was done by use of the comparative threshold cycle (Ct) method, where the Ct value is the cycle number at which the fluorescence signal of the amplified target reaches a defined threshold (Schmittgen and Livak 2008). Ct data on the transcripts of interest were normalised by subtraction of the Ct value of 18 s ribosomal RNA to obtain the ΔCt value. To obtain normal distributed data, $2^{-\Delta Ct}$ values were used for statistical analysis.

Kidney transporter protein-level measurements

Frozen whole kidneys were thawed on ice and homogenised with a Yellowline DI25 Basic Homogeniser (Bie & Berntsen, Glostrup, Denmark) in an ice-cold buffer containing 300 mM sucrose, 25 mM imidazole, 1 mM EDTA, and 1:200 Calbiochem Protease inhibitor cocktail set II, EDTA-Free (prod. no. 539134, Calbiochem, Darmstadt, Germany). Protein determination and Western blotting were conducted as previously described (Hadrup et al. 2007) except that the employed primary antibodies were anti-organic anion transporter 1 (OAT1) (prod. no. ABIN653184, Antibodies-online, Aachen, Germany), anti-organic anion-transporting polypeptide 1/3 (OATP1/3) (prod. no. Sc-47265, Santa Cruz Biotechnologies, La Jolla, CA) and anti-organic anion-transporting polypeptide 4C1 (OATP4C1) (prod. no. Sc-136775, Santa Cruz Biotechnologies, La Jolla, CA). All antibodies were used at a dilution of 1: 2,000. Moreover, the employed camera was a Gel Doc 2000 (Bio-Rad, Hercules, CA). To accommodate up to 36 samples, a total of three blots were run for each investigated PFNA dose level (0.0125 mg/kg bw/day (PFNA Low) and 0.25 mg/kg bw/day (PFNA Mid) and on each blot a standard sample with a pooled volume of eight randomly selected samples was included, and all samples on each blot were normalised to this sample before data analysis.

Statistics

Regarding data on mechanisms of toxicity (hormone concentrations, mRNA data, protein-level data), only the Low and Mid PFNA doses were included in the statistical evaluation (and on the graphs). The High PFNA groups were excluded due to the severe toxicity observed on body weight and pathology. Data were analysed for normal distribution by use of the D'Agostino & Pearson omnibus normality test. In case of a lack of normal distribution, data were transformed using the logarithm function and again tested for normal distribution. For normally distributed data, one-way ANOVA with Dunnett's post-test was employed to assess effects of PFNA and of PFNA + Mix, respectively. In case of lack of normality, the nonparametric Kruskal–Wallis test with Dunn's post-test (exposed groups vs. controls) was employed. To assess differences between individual data points, a *t* test was employed. In case of lack of normality, differences between two groups were assessed by use of the Mann–Whitney test. In case of comparison with a group in which all data were below the limit of detection, one sample *t* test was employed. A *p* value of <0.05 was considered significant. The statistical software package was Graph Pad Prism (Graph Pad Software, La Jolla, USA).

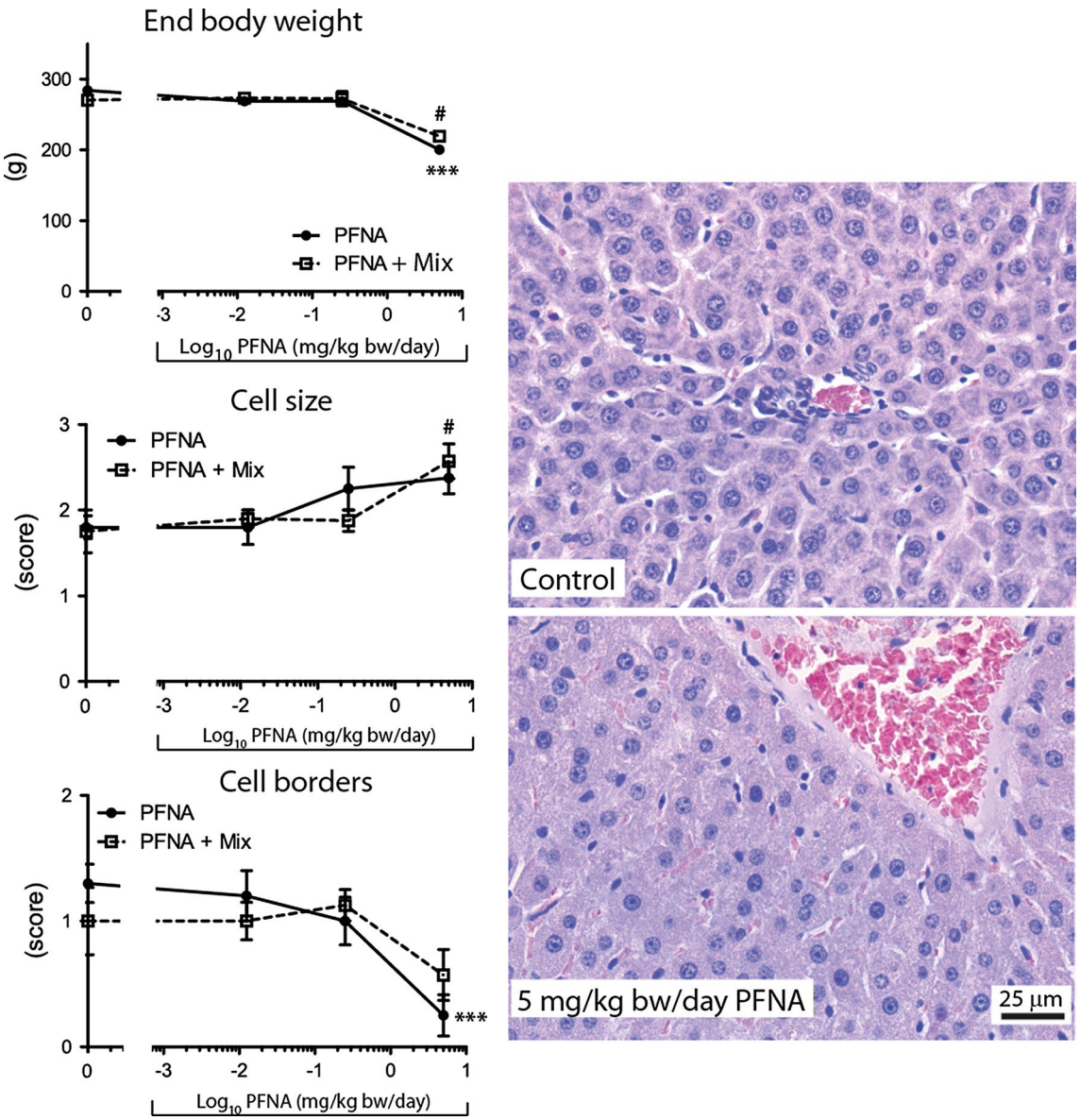


Fig. 2 At high-dose, PFNA induces toxicity as measured by body weight and pathology. Rats were administered PFNA at 0.0125, 0.25 or 5 mg/kg bw/day for 14 days in the presence or absence of a mixture of 14 chemicals (Mix) at a total dose of 2.5 mg/kg bw/day. A piece of liver was fixed in paraformaldehyde and processed into paraffin-embedded sections and stained with haematoxylin and eosin. Sections were then blinded to the observer and scored by use of microscopy. *Upper* image shows a section from a control rat, *lower* image shows a section from a rat administered 5 mg/kg bw/day PFNA. The *upper* graph shows that the end body weight is increased at High PFNA ± Mix. The *lower* graphs show scores of cells size and cell borders. Cell size is increased by High PFNA ± Mix, and cell borders are decreased by High PFNA ± Mix. Data are mean *plus* or *minus* SEM. *N* was 10 except for Mid PFNA, Mix and Mid PFNA + Mix where *n* was 8. Data are analysed by one-way ANOVA or Kruskal–Wallis test. ***$p < 0.001$ by Dunnett's ANOVA post-test for PFNA versus control; and $^{\#}p < 0.05$ by Dunn's Kruskal–Wallis post-test for PFNA + Mix versus control

Results

PFNA plasma concentration

PFNA plasma concentrations following administration of 0.0125 mg/kg bw/day were 396 ng/mL for Low PFNA and 1,111 ng/mL for Low PFNA + Mix—a difference that was statistically significant ($p = 0.0007$). Following dosage with 0.25 mg/kg bw/day, the plasma concentrations were 29,950 ng/mL (Mid PFNA) and 39,880 ng/mL for Mid PFNA + Mix. Following dosage with 5 mg/kg bw/day, the values were 602,000 ng/mL (High PFNA) and 541,700 ng/mL for High PFNA + Mix (Fig. 1). Thus, at the low PFNA dose, the Mix caused a pronounced increase

in PFNA plasma levels, but this did not happen at higher PFNA doses.

Body weight, organ weights and pathology

General toxicity was observed with High PFNA with and without Mix. The end body weights were decreased in High PFNA and High PFNA + Mix groups (Fig. 2). Macroscopic pathological examination showed steatotic livers and congestive hearts (pictures not shown) that were observed for High PFNA and High PFNA + Mix. Microscopically, increased size of liver cells (hypertrophy) was observed with increasing doses of PFNA, reaching statistical significance in the High PFNA + Mix group (Fig. 2). Cell borders were less apparent in liver sections of rats receiving increasing doses of PFNA being statistically significant in the High PFNA group.

Hormone plasma concentrations

The corticosterone plasma concentration was increased at the Low PFNA dose level without Mix (twofold). In the presence of the Mix, corticosterone was not increased at the Low and Mid PFNA groups (Fig. 3). For androstenedione, testosterone and dihydrotestosterone increases were found in the Low PFNA + Mix group as compared to Low Mix (Fig. 3). For androstenedione and testosterone, a decrease was found both in the High PFNA and the High PFNA + Mix groups (data not shown). This effect was not detected for dihydrotestosterone for which many measurements were below the level of quantification (data not shown). Regarding pituitary hormones (Fig. 4), Mid PFNA + Mix decreased the plasma concentration of luteinising hormone (LH). The concentration of follicle stimulating hormone (FSH) was decreased by Low PFNA + Mix and adrenocorticotropic hormone (ACTH) was decreased in the Mid PFNA + Mix as compared to Mid PFNA. For prolactin and brain-derived neurotrophic factor, PFNA showed higher concentrations as compared to PFNA + Mix at the Mid dose.

mRNA levels in testis, liver and fatty tissue

In the testes, 17β HSD was down-regulated at both Low and Mid PFNA + Mix as compared to control. This effect was not observed with PFNA alone (Fig. 5). For all other genes measured in testes, there were no significant effects at the mRNA levels of genes involved in regulation of steroid metabolism when considering Low PFNA and Mid PFNA with or without Mix (data not shown). At the High PFNA, where severe toxicity was found, mRNA levels of steroidogenic acute regulatory protein (StAR), benzodiazepine receptor (BZRP), CYP11A, CYP17 and 17β-hydroxysteroid dehydrogenase (17β HSD) were all found to be down-regulated by PFNA (data not shown).

Fig. 3 PFNA at a dose of 0.0125 mg/kg bw/day plus Mix induces an effect on androgens. Rats were administered PFNA at 0.0125or 0.25 mg/kg bw/day for 14 days in the presence or absence of a mixture of 14 chemicals (Mix) at a total dose of 2.5 mg/kg bw/day. Steroid hormones were measured in plasma by use of LC–MS/MS. Low PFNA + Mix increased testosterone and dihydrotestosterone. A normalising effect was seen on corticosterone with Low PFNA + Mix. Data are mean plus or minus SEM. *N* was 10 except for Mid PFNA, Mix and Mid PFNA + Mix where *n* was 8. Data were analysed by one-way ANOVA or Kruskal–Wallis test. $^{**}p < 0.01$ by Dunnett's ANOVA post-test for PFNA versus control. $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ by *t* test for PFNA versus PFNA + Mix. For dihydrotestosterone, a one sample *t* test was applied versus the detection limit of this hormone. $^{\S\S}p < 0.01$

In the liver, 11β HSD was down-regulated with PFNA + Mix at both Low and Mid doses. This effect was not observed for PFNA alone (Fig. 5). For all other genes, no effects were found at the Low and Mid PFNA groups. At the High PFNA groups, where severe toxicity occurred, the aldo–keto reductase family 1 member C1 (AKR1C1), UDP-glucuronosyltransferase 2B15 (UGT2B15), CYP2C11, CYP1A2 and CYP2B6 were all found to be down-regulated, whereas CYP3A23/3A1 was found to be up-regulated (data for the high-dose groups are not shown on the graphs).

In adipose tissue, CYP19 mRNA was up-regulated for Low PFNA + Mix as compared to Low PFNA (Fig. 5). At the toxic High PFNA, CYP11 was up-regulated (data not shown). There were no effects on the other steroid metabolism enzymes: CYP17, 17β HSD and 5α reductase (data not shown).

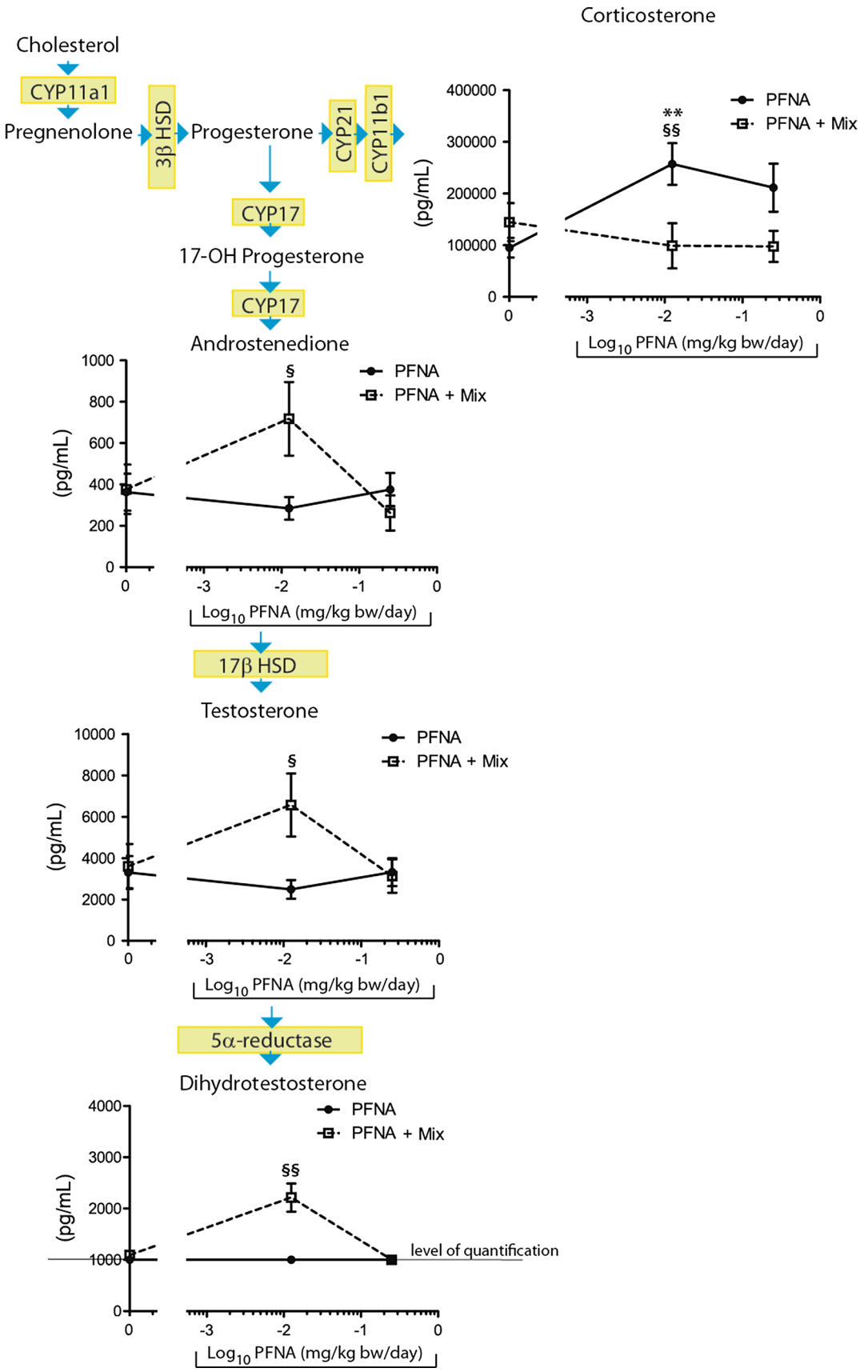
Kidney transporter protein levels

OAT1 was increased in whole kidney homogenates when Low PFNA was combined with Mix (Fig. 6). OATP4C1 was decreased by Low PFNA alone. This effect was normalised by addition of Mix (Fig. 6). At the Mid dose level, no significant effects were found. OATP1/3 was not affected at any of the investigated dose levels (data not shown).

Discussion

Relevance of the employed PFNA doses in relation to human exposure levels

The hypothesis of the current study was that adverse mixture effects occur at doses approaching high-end human exposure levels of food chemicals. Our aim was to reach a plasma level of PFNA covering the combined human


Cholesterol
CYP11a1
Pregnenolone
3β HSD
Progesterone
CYP21
CYP11b1
CYP17
17-OH Progesterone
CYP17
Androstenedione
17β HSD
Testosterone
5α-reductase
Dihydrotestosterone
Corticosterone
PFNA
PFNA + Mix
(pg/mL)
Log10 PFNA (mg/kg bw/day)
**
§§
§
level of quantification

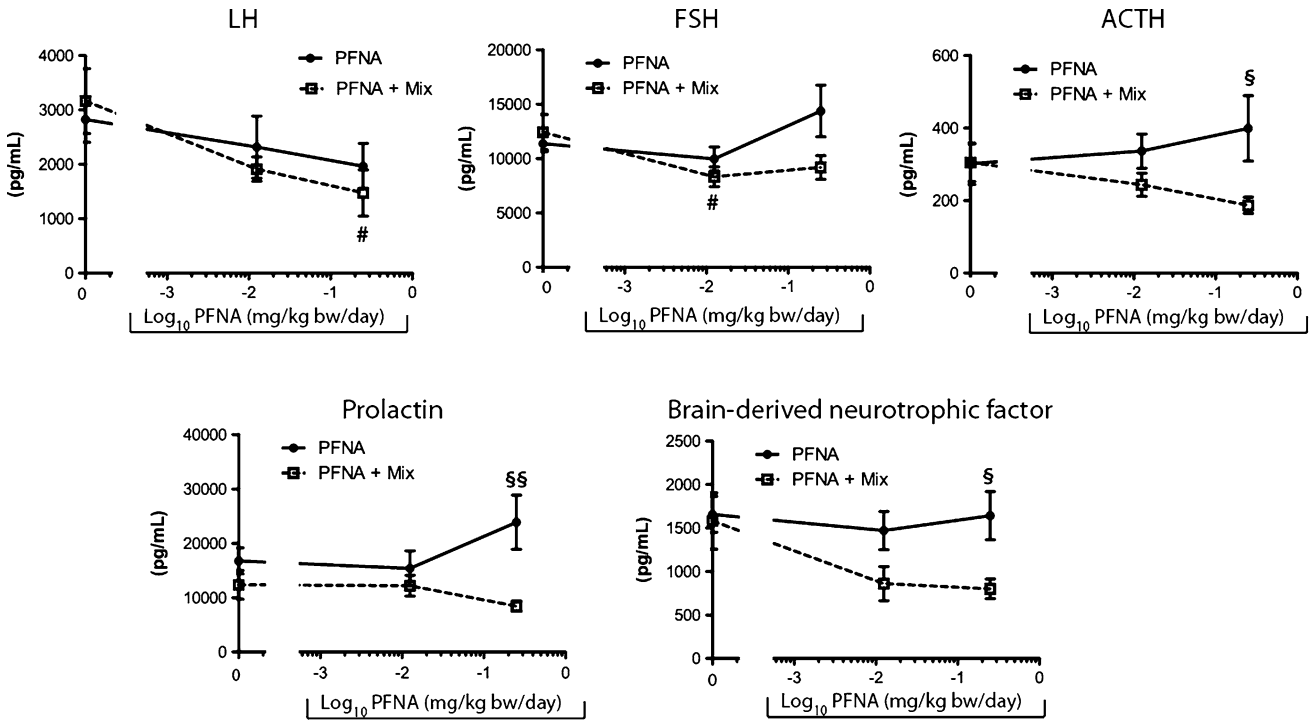


Fig. 4 Effects of dose PFNA and Mix on pituitary hormone levels. Rats were administered PFNA at 0.0125 or 0.25 mg/kg bw/day for 14 days in the presence or absence of a mixture of 14 chemicals (Mix) at a total dose of 2.5 mg/kg bw/day. Hormones were measured by use of a Milliplex Map Rat Pituitary Luminex Kit. LH was decreased by Mid PFNA + Mix. FSH was decreased by Low PFNA + Mix. For ACTH, prolactin and brain-derived neurotrophic factor Mid PFNA + Mix was lower than Mid PFNA alone. Data are mean *plus* or *minus* SEM. N was 8 except for control ($n = 10$) and Low PFNA ($n = 9$). Data were analysed by one-way ANOVA. $^{\#}p < 0.05$ by Dunnett's ANOVA post-test for PFNA + Mix versus control. $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ by *t* test for PFNA versus PFNA + Mix

plasma concentrations of perfluorinated compounds such as PFOA, PFOS and PFNA. According to Lau et al. (2007), human exposure means obtained from a substantial number of investigations gave a combined value of these three perfluorinated compounds of 13–429 ng/mL (or 13–117 ng/mL if the second highest PFOA value is used). For comparison, the 95th percentile obtained from the NHANES study was 67.6 ng/mL for PFOA, PFNA and PFOS combined (CDC 2009) which is in line with the Lau et al. data. A PFNA plasma concentration of 396 ng/mL was found in the Low PFNA group, indicating that PFNA in this study may be up to a factor of six higher than a high-end human exposure level (Table 1). However, this depends on the choice of a 'high-end' exposure for which a fixed, true value do not exist. In a study, not reported in the review by Lau et al., Emmett et al. found a mean of 423 ng/mL PFOA for persons not subjected to occupational exposure (median 329, $n = 312$) and a mean of 824 ng/mL in persons with substantial occupational exposure (median 775, $n = 18$) (Emmett et al. 2006). Taking this into account, the present PFNA exposure in the rats was close to a combined human PFNA, PFOA and PFOS exposure. We made the assumption that the applied PFNA exposure should represent exposure to other perfluorinated congeners, although it can be argued that the difference in structures of these molecules affects potency and binding specificity to different target molecules in the mammalian body. It is noted that a range of congeners different from PFNA, PFOA and PFOS exist, and these likely also contribute to the combined effects of perfluorinated compounds in humans.

Observed mixture effects and discussion on the presence of interactions

The aim of the present investigation was to test whether a relevant mixture of food chemicals at doses approaching high-end human exposure levels exerted adverse effects. The study was designed to target testosterone, and we found this steroid as well as its downstream metabolite, dihydrotestosterone and its precursor, androstenedione, to be affected by the mixture. There were no significant effects of Low PFNA or of Mix alone, whereas androgen levels increased considerably after exposure to Low PFNA in combination with Mix; thus for testosterone, dihydrotestosterone, and androstenedione a non-monotonic effect seemed to have occurred. Previously, additive mixture effects have

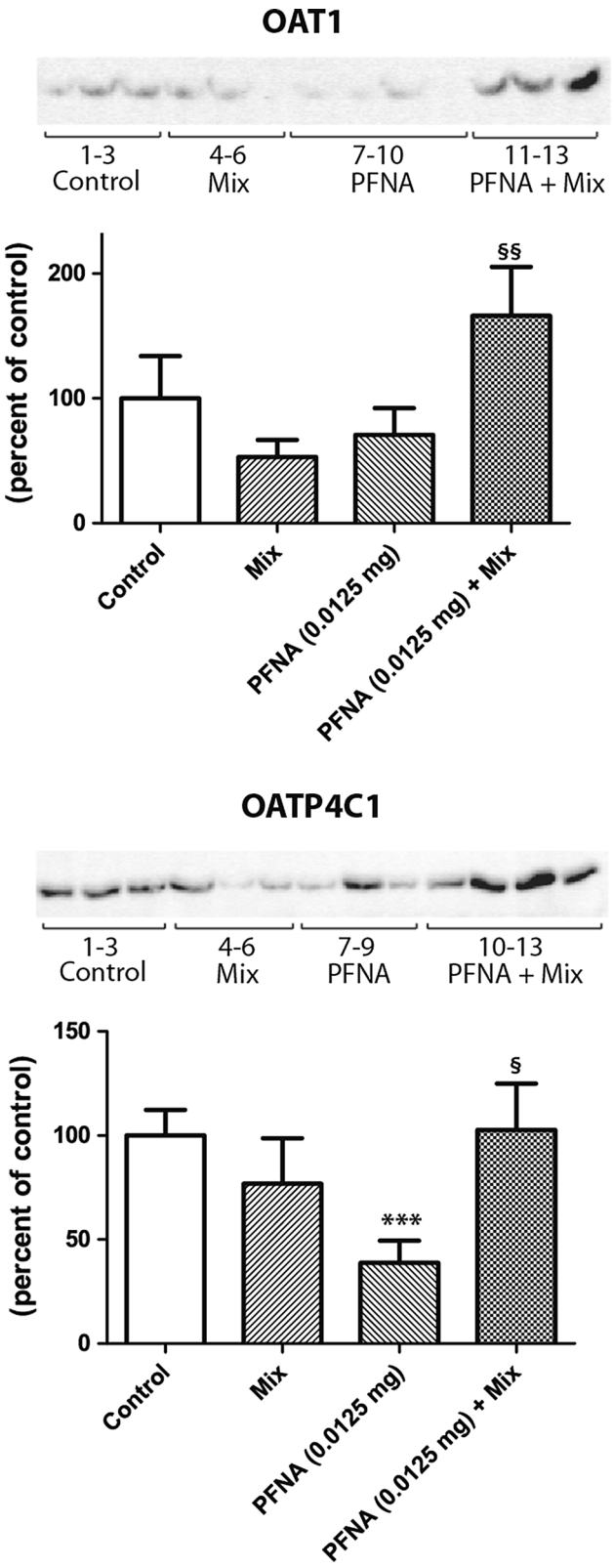


Fig. 6 Effects of low-dose PFNA and Mix on protein levels of organic anion transporter 1 and organic anion-transporting polypeptide. Rats were administered PFNA at 0.0125 mg/kg bw/day for 14 days in the presence or absence of a mixture of 14 chemicals (Mix) at a total dose of 2.5 mg/kg bw/day. Protein levels of OAT1 (organic anion transporter 1) and OATP4C1 (organic anion-transporting polypeptide-4C1) were measured by Western blotting. All samples were measured in a total number of three blots per protein. Only one blot is shown on the figure. A standard sample on each blot was used for normalisation to obtain a graph representing all samples ($n = 9$ for control, $n = 8$ for Mix, $n = 10$ for Low PFNA (0.0125 mg/kg bw/day) and $n = 10$ for Low PFNA + Mix). OAT1 was increased at Low PFNA + Mix as compared to Low PFNA. OATP4C1 was decreased at Low PFNA as compared to control, and this effect was normalised at Low PFNA + Mix. Data are mean plus or minus SEM. Data were not normal distributed and were therefore tested by use of the nonparametric Mann–Whitney test. ***$p < 0.001$ for PFNA versus control. $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ for PFNA versus PFNA + Mix

been found for chemicals each present at doses for which measurement techniques are not sensitive enough to detect their individual effects (Hass et al. 2007; Silva et al. 2002). Feng et al. have previously demonstrated a non-monotonic dose–response curve of PFNA on testosterone in rats with an increase at 1 mg/kg bw/day and a decrease at 5 mg/kg bw/day. This supports the presence of such a non-monotonic relationship although this was seen at higher-dose levels. Also Wade et al. (2002) found a non-monotonic dose–response effect on natural killer cell lytic activity following exposure to a mixture containing 18 persistent contaminants at doses ranging from 1× to 100× the estimated safe level.

Looking at the PFNA plasma concentration, a toxicokinetic interaction was found in that the PFNA plasma concentration was increased at Low PFNA + Mix as compared to Low PFNA. PFNA could not be detected in animals only given Mix, indicating that no background PFNA levels, e.g., from the feed were present in the animals. Thus, the 14-chemical mixture is able to increase the PFNA plasma levels at lower doses possibly by interference with ADME issues for PFNA. For corticosterone plasma levels, Low PFNA + Mix normalised an increase in corticosterone observed for Low PFNA. Also on the kidney OATP4C1 transporter, the addition of Mix to Low PFNA normalised a Low PFNA-induced decrease in the protein level. These data suggest that in addition to reported food–drug interactions (Bailey et al. 1989), food–environment chemical interactions may occur at the toxicokinetic level at doses approaching high-end human exposure levels. The effect of Mix on PFNA plasma levels is due to a toxicokinetic interaction between Mix and PFNA, but whether the effect on steroid hormone levels is due to kinetic or dynamic interferences is unknown. It is striking that we have detected potential non-monotonic low-dose effects. This gives food for thought concerning the extrapolation from high to low doses typically done in toxicological studies and concerning future human risk assessment of chemicals.

In this study, possible effects of a chemical mixture were investigated in juvenile male rats 7 weeks of age at onset. Male Wistar rats are considered sexually mature when they

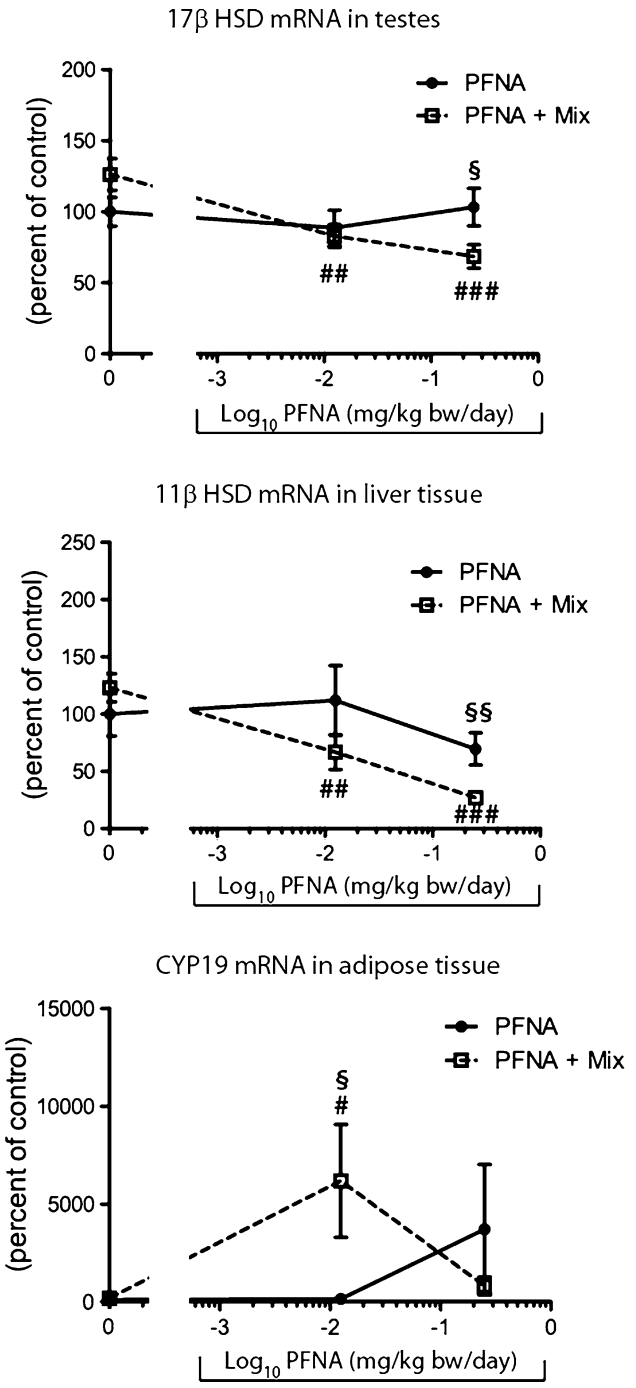


Fig. 5 mRNA levels of 17β HSD in testes, 11β HSD in liver tissue and CYP19 in adipose tissue. Rats were administered PFNA at 0.0125 or 0.25 mg/kg bw/day for 14 days in the presence or absence of a mixture of 14 chemicals (Mix) at a total dose of 2.5 mg/kg bw/day. mRNA levels were measured by use of qPCR. 11β and 17β HSD were decreased by Low and Mid PFNA + Mix. CYP19 was higher for Low PFNA + Mix as compared to Low PFNA. Data are mean *plus* or *minus* SEM. N was 10 except for Mid PFNA, Mix and Mid PFNA + Mix where n was 8. Data were analysed by one-way ANOVA. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ by Dunnett's ANOVA post-test for PFNA + Mix versus control. $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ by *t* test for PFNA versus PFNA + Mix

are around 50 days old; thus, we have investigated effects in rats just post-puberty. In our view, marked hormone changes may be considered as adverse. Increased testosterone in males has for example been linked to increased aggression (McGinnis 2004); and androgenic anabolic steroid abuse has been linked to cardiac disease (Ahlgrim and Guglin 2009).

Possible mechanisms involved in the mixture effects on androgens

The possible mechanisms underlying the observed mixture effects could provide a deeper understanding of how such effects develop in the mammalian body. Regarding the mixture effects on androgens, mechanisms involved could be related to (a) increased production or release of steroid hormones either via hormonal factors or locally induced (b) decreased tissue deposition of hormones (c) decreased metabolism of androgens and/or (d) decreased excretion of hormones. The pituitary hormone LH is involved in the regulation of testosterone production in the testes, and LH was decreased by Mid PFNA + Mix, and this may reflect a secondary negative feedback on the pituitary caused by disturbed steroid hormone levels.

In the testes, there were no findings to explain the effects seen on androgens except that a disturbed 17β HSD mRNA regulation may be part of the explanation. In adipose tissue, CYP19 was increased at Low PFNA + Mix as compared to Low PFNA. This suggests increased conversion of testosterone to estradiol when Mix is present. Among the chemicals in Mix, only DDE has shown a link to CYP19. Daughters of Michigan fish-eaters had increased gene expression of CYP19 in blood leucocytes that was correlated to prenatal DDE levels (Karmaus et al. 2011). The fact that CYP19 was increased in the current study indicates that a compensatory mechanism to eliminate the excess testosterone might be in play.

In the liver, CYP expression was investigated because CYP3A4 and CYP2C9 (CYP3A23/A1 and 2C11in rats) are involved in the hydroxylation of testosterone, and these enzymes are known to be inhibited by bergamottin and glabridin constituents of the Mix (Cheng and Schenkman 1983; Guengerich 1999; Kenworthy et al. 1999; Martignoni et al. 2006; Tassaneeyakul et al. 2000; Yamazaki and Shimada 1997). Moreover, in the ToxCast (high throughput in vitro screening project) several perfluorinated carboxylic acids including PFNA as well as BPA and prochloraz (constituents of the Mix) have been found in vitro to inhibit CYP2C9 (US_Environmental_Protection_Agency 2014). However, no effects on the mRNA levels of CYP3A23/1 or CYP 2C11 for Low PFNA in combination with Mix were found. Also, on the steroid glucuronidation pathway, there were no findings to explain the effects seen on androgens.

To study interaction via excretion, we investigated organic anion transporters in the kidney because these transporters have been suggested to play a role in PFNA and testosterone excretion. OAT1 as well as OATP4C1 transport organic anions from the blood into the kidney tubular cells for subsequent secretion into the pre-urine (Han et al. 2012). With Low PFNA + Mix, an increase in protein level of OAT1 was found (Fig. 6) but neither Mix nor PFNA individually exerted this effect. It has been shown that testosterone stimulates OAT1 (Cerrutti et al. 2002; Ljubojevic et al. 2004) thus the increased testosterone level seen at Low PFNA + Mix correlates well with an increase in this transporter. This up-regulation, however, might also reflect that OAT1 could transport PFNA and that this transporter is up-regulated as a compensatory mechanism in order to excrete the increased amounts of PFNA. For OATP4C1, the Mix normalised the Low PFNA-induced decrease in the protein level, suggesting that the Mix protects the body by allowing an increased transport of PFNA (Fig. 6). As for OAT1, OATP4C1 is increased by testosterone (Lu et al. 1996), again suggesting the increased testosterone level to be causing the normalisation of the OATP4C1 expression. Taken together, the data on kidney transport do not readily explain the mixture effect on androgens at Low PFNA + Mix and may rather reflect downstream effects of the increased androgen levels.

Possible mechanisms underlying the increased PFNA concentration caused by Mix

The increase in the PFNA plasma concentration following Low PFNA + Mix as compared to Low PFNA may be explained by (1) increased PFNA absorption, (2) decreased metabolism, (3) decreased tissue deposition, or (4) decreased excretion. We looked for metabolites of PFNA but did not find any significant metabolism, and this is in accordance with the literature (Lau et al. 2007). Regarding proposed reabsorption of perfluorinated compounds, the OATP1 is located on the luminal side of the tubular cells (Han et al. 2012) and has been demonstrated to have the ability to transport perfluorocarboxylates (Yang et al. 2009b). OATP1 was not found being affected by Low PFNA alone or in combination with Mix; thus, testosterone-stimulated reabsorption of PFNA via this transporter seems not to be underlying the increased plasma concentration of PFNA. However, it should be noted that we did not measure two other OATs involved in perfluorocarboxylate transport, namely OAT2 and OAT3 (Kudo et al. 2002). Whether the PFNA plasma-level effect is a cause or a consequence of (or not related to) the androgen levels is unknown. However, when we depict the effect on androgen levels as a function of the internal PFNA plasma levels (graph not shown), we still observe a pronounced increase in androgen levels, indicating that there is more to the androgen effect than just affected toxicokinetics by PFNA alone.

Possible mechanisms underlying the normalisation of corticosterone

The mechanism underlying the normalisation of the corticosterone level by the Mix is likely explained by the diminished plasma ACTH observed when Mix was administered along with PFNA. Thus, a central effect on the pituitary may be evident. The enzymes responsible for inter-conversion of corticosterone to the physiologically inactive 11-dehydrocorticosterone in rats are 11β HSD (Thomson et al. 1998). We found PFNA + Mix to down-regulate this enzyme at the mRNA level. A differential regulation of this enzyme may be an explanation of the observed differential effect on corticosterone caused by PFNA with or without Mix.

Mixture-independent effects of PFNA suggest non-monotonic dose–response relationships

At Low PFNA dose, an increased corticosteroid effect as well as a diminished effect of the OATP4C1 protein level in kidney was observed (Fig. 6), suggesting that this chemical exerts low-dose effects on its own. The PFNA effect on corticosterone plasma levels could involve regulation of 11β HSD. PFNA has shown a potential to activate liver X receptor α (LXRα) (US_Environmental_Protection_Agency 2014), and LXR negatively regulates expression of 11β HSD (Vogeli et al. 2013). At High PFNA, macroscopical and microscopical pathology of the liver as well as decreased body and organ weights showed that this dose is highly toxic to the animals. From that point of view, it is not surprising that the mRNA levels of several enzymes are down-regulated as seen for testes and liver, along with the testosterone and androstenedione plasma concentration being down-regulated at the high dose. Several enzymes like StAR, BZRP and CYP11A in the testes were down-regulated and provide a suggestion for the decrease in testosterone and androstenedione at high doses. Steatosis, obscure hepatic cell borders and a decrease in testosterone have been shown for perfluorinated compounds by others previously (Fang et al. 2010; Feng et al. 2009; Yang et al. 2009a). Notably, when these toxic effects occur, CYP1A2, CYP2B6 and CYP2C11 were down-regulated by PFNA, whereas CYP3A23/3A1 corresponding to the human CYP3A4 was up-regulated suggesting that this CYP is up-regulated to increase elimination of multiple toxic metabolites and confirming the broad substrate specificity of this enzyme. Our findings highlight the challenge of extrapolating from high-dose to low-dose effects, as is the ordinary

practice within toxicology and suggest that lower doses should be employed as well in toxicological studies.

Conclusion

We found that a human relevant mixture of fifteen chemicals given to rats at doses approaching human realistic high-end exposure levels disturbed several plasma steroid and pituitary hormone levels. Androgen levels were non-monotonically increased by >100 %, and corticosterone levels were decreased by 60 %. Moreover, a toxicokinetic interaction may have occurred as the Mix caused a markedly increased PFNA plasma concentration. Our data suggests that mixture effects of chemicals may be non-monotonic and may occur even at doses approaching high-end human exposure levels. Further studies are warranted to determine whether this reflects a general phenomenon that should be taken into consideration when predicting human mixture toxicities.

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Conflict of interest The authors declare that there are no conflicts of interest.

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Paper III: Exposure to perfluorononanoic acid combined with a low-dose mixture of 14 human-relevant compounds disturbs energy/lipid homeostasis in rats

Metabolomics

Aim: Increasing the information obtained from toxicological studies by profiling of the plasma metabolite composition and hepatic gene expression; focusing on low dose effects of toxic chemicals on the metabolome.

Results: The metabolomics profiling displays a decrease in the phospholipids and neutral lipids. The mixture reduced mono- and diacylglycerols in the plasma. The primary effect is driven by the mixture. The transcriptomics profiling displays effect on PPAR mainly driven by PFNA.

Conclusion: A low dose of chemicals affects the metabolome and the transcriptome. The mechanism of action can be difficult to determine though both mixture and low PFNA seemed to have effect on the lipid metabolism of the rat. The transcriptomics data suggests that PFNA have effect on the lipid metabolism by affecting PPAR.

Metabolomics
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ORIGINAL ARTICLE

Exposure to perfluorononanoic acid combined with a low-dose mixture of 14 human-relevant compounds disturbs energy/lipid homeostasis in rats

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Abstract Humans are constantly exposed to a significant number of compounds and many are readily detected in human body fluids. Worryingly, several of these compounds are either suspected to be, or have already been shown to be harmful to humans either individually or in combination. However, the potential consequences of low-dose exposure to complex mixtures remain poorly understood. We have profiled the effects on rat blood plasma and liver homeostasis using metabolomics and transcriptomics following 2-week exposure to either a mixture of 14 common chemicals (Mix), perfluorononanoic acid (PFNA) at low (0.0125 mg/kg/day) or mid (0.25 mg/kg/day) doses, or a combination of Mix and PFNA. In blood plasma, 63 and 64 metabolites were significantly changed upon exposure to Mix alone or PFNA + Mix, respectively. Twelve of the metabolites were identified and comprised mainly lipids, with various lipid classes differentially affected across study groups. In the liver, expression of 182 and 203 genes—mainly related to energy homeostasis and lipid metabolism—were differentially expressed upon exposure to PFNA alone or PFNA + Mix, respectively. In general, Mix alone affected lipid metabolism evident in blood plasma, whereas effects on lipid metabolism in the liver were mainly driven by PFNA. This study verifies that a chemical mixture given at high-end human exposure levels can affect lipid homeostasis and that the combined use of metabolomics and transcriptomics can provide complimentary information allowing for a detailed analysis of affected signaling pathways.

Keywords Perfluorononanoic acid · Metabolomics · Transcriptomics · Lipid homeostasis · Mixture toxicology

1 Introduction

Humans, particularly those living in industrialized countries, are continuously exposed to a plethora of compounds through foods, cosmetics, pharmaceuticals, air inhalation and more (Monosson 2005). A large number of studies have reported on the presence of multiple compounds in human body fluids (Calafat et al. 2007; NHANES 2013), clearly showing that they are taken up by the body through various routes. Also, epidemiological studies have shown strong associations between compound mixtures and diseases, for instance in relation to human reproduction (Krysiak-Baltyn et al. 2012; Taylor et al. 2014). Therefore, since animal studies have shown effects of human relevant mixtures of environmental compounds given at doses close to No Observed Adverse Effect Levels (NOAELs) for single compounds (Christiansen et al. 2008, 2012; Axelstad et al. 2014), the presence of many of these chemicals simultaneously is of real concern to human health.

The traditional approach for toxicological testing aims at understanding the effect(s) of a single compound on biological systems, from cells (in vitro) to animals

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(in vivo). The compound is typically investigated at varying doses in order to obtain information on parameters such as the NOAEL. A few studies on rats exposed to chemicals or chemical mixtures at doses representing human exposure levels have been reported (Moser et al. 2006; Chen et al. 2014; Hadrup et al. 2015). These studies suggest that even at low doses, certain compounds can have effects on biological systems, for instance the plasma metabolome. However, there still is a significant gap in knowledge regarding effects of low-dose chemical mixtures on either the metabolome or transcriptome at the 'omics' level.

'Omics' technologies have provided new approaches to evaluate the effects of toxic compounds. For instance, fluorinated compounds have been shown to change the lipid metabolism of both rats and zebrafish (Fang et al. 2012a; Zhang et al. 2012) and endocrine disrupting compounds such as bisphenol A have been shown to cause changes to the metabolome even at doses far below the NOAEL (Chen et al. 2014). In general, changes in the transcriptome and metabolome are detectable even after exposure to a low dose of certain compounds, which suggests that these methods can be valuable tools for understanding how the compounds affect the organism where obvious phenotypes are absent (Chen and Kim 2013). Furthermore, it has been shown that a mixture of compounds at low doses can result in a marked effect even when individual compounds show no detectable effect and that this effect is marked even if they 'only' act additively (Silva et al. 2002).

We wanted to investigate the effects of a mixture of compounds (Mix) alone and together with increasing doses of the perfluorocarboxylic acid, perfluorononanoic acid (PFNA), as well as the effects of PFNA alone. The Mix was composed of 12 environmentally relevant endocrine disrupting compounds at high-end human exposure levels (Christiansen et al. 2012) and two food ingredients in doses corresponding to a high-end daily intake of grapefruit and licorice. We hypothesized that adverse effects would be observed with Mix alone or when combined with low dose PFNA. By use of two complementary omics methods, metabolomics and transcriptomics, we profiled the effects in blood plasma and livers of exposed rats and compared them to adverse effects observed at higher PFNA doses. We found significant changes both to the metabolome, as detected in plasma, and the liver transcriptome following exposure to Mix and PFNA.

2 Materials and methods

2.1 Compounds and dosing

Compounds were selected as previously described (Hadrup et al. 2015). Animals were dosed with a mixture of 12 compounds: bisphenol A, butylparaben, dibutyl phthalate (DBP), bis(2-ethylhexyl)phthalate (DEHP), 4-methylbenzylidene camphor, octyl methoxycinnamate, dichlorodiphenyldichloroethylene (p,p'-DDE), epoxiconazole, linuron, prochloraz, procymidone, vinclozolin) and described elsewhere (Christiansen et al. 2012; Hadrup et al. 2013), along with the two food components; glabridin from licorice and bergamottin from grapefruit (Hadrup et al. 2015), both known to inhibit the activity of cytochrome P450s (CYPs) metabolizing hormones and chemicals. The total dose of the Mix was 2.5 mg/kg/day and the ratio of the compounds is presented in Supplementary Table 1. The dose of Mix was based on previously observed endocrine disruption (Christiansen et al. 2012), albeit 24-times lower herein to reflect human exposure corrected for different body surface areas of rat and human. In addition to Mix, animals were exposed to three different doses of PFNA, the lowest dose corresponding to a high-end human exposure level (Lau et al. 2007). The two lowest doses were Low = 0.0125 mg/kg/day and Mid = 0.25 mg/kg/day. A high dose of PFNA (5 mg/kg/day) was part of the study as well but was excluded from the mechanistic analyses in this paper due to the observed severe toxicity (Hadrup et al. 2015) that would hamper the interpretation of data.

2.2 Animals

The animal study has been described previously (Hadrup et al. 2015). In brief, male Wistar Hannover Galas rats at 6 weeks of age were housed two per cage with a 12-h light/dark cycle and ad libitum access to acidified tap water and standard diet. The animals received vehicle (corn oil) or test substances once daily by gavage for 14 days. Fifty-four rats were randomly assigned into six groups (Table 1). The last dose was administered to each animal 75–105 min before euthanization. The rats were anaesthetized in CO_2/O_2 prior to decapitation. Plasma was isolated from heparinized neck blood by centrifugation at 1000*g* at 4 °C for 10 min and subsequently stored at −80 °C. Livers were weighed and snap-frozen in liquid nitrogen.

2.3 Metabolomics

The procedure was as previously described (Skov et al. 2014). In brief, phospholipids were adsorbed on a

Table 1 Acronyms used for the different treatment groups and respective number of animals per group

	Without Mix	With Mix	Acronym
Control	10	8	Control/mix
0.0125 mg/kg/day	10	10	Low PFNA ± mix
0.25 mg/kg/day	8	8	Mid PFNA ± mix

phospholipid SPE column (Supelco, Sigma-Aldrich). The eluate was collected, dried and extracted using first 200 µl heptane to isolate the lipids followed by 200 µl methanol to extract the more polar compounds. The phospholipids were eluted from the SPE column using 300 µl 10 % NH_4OH in methanol. The phospholipid, lipid and the polar fractions were analyzed by an HPLC system combined with a Maxis Quadrupol Time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany).

Data were analyzed with Profile Analysis 2.1 (Bruker Daltonics, Bremen, Germany). Data were extracted using the "find molecular features" algorithm in a mass range from 50 to 1100 m/z value. The noise was reduced by removing peaks that were present in <50 % of the samples among all treatment groups and at the same time had a peak intensity of ≤3000. The calculations were carried out in R (R Core Team 2012). The data were uploaded to MetaboAnalyst.ca (Xia et al. 2012) and analyzed with *t* test, principal component analysis, and partial least squares discriminant analysis (PLS-DA). The accurate masses of significantly different metabolites were searched for in databases such as the human metabolome database (HMDB) (Wishart et al. 2009). The identities of the compounds were verified by comparison of MS/MS patterns with data from the databases HMDB, METLIN (www.metlin.scripps.edu) (Smith et al. 2005), LIPID MAPS (www.lipidmaps.org) (Sud et al. 2007) and MassBank (www.massbank.jp) (Horai et al. 2010).

2.4 Statistical analysis

Initial analyses of the metabolome data were performed on the Metaboanalyst server (Xia et al. 2012). Here, a one-way analysis of variance (ANOVA) comparing PFNA-treated animals to control animals and PFNA + Mix-treated animals to control, respectively, formed the basis for initial selection of significantly altered metabolites. All *p*-values were adjusted using false discovery rate (FDR) according to the protocol implemented in the Metaboanalyst workflow, and 0.05 was used as cut-off for statistical significance. The statistically significantly altered metabolites were subsequently analyzed and plotted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The D'Agostino and Pearson omnibus normality test was used to test for normality of the data. If data were normally distributed, an ANOVA was performed using Dunnett's multiple comparisons test to adjust the *p*-values. If data were not normally distributed, data were log-transformed and, if normally distributed after transformation, analyzed by ANOVA. If not normally distributed, a non-parametric Kruskal–Wallis test followed by a Dunn's multiple comparisons test was conducted. The criteria for statistical significance was $p < 0.05$, $p < 0.01$ and $p < 0.001$ denoted *, ** and ***, respectively. Statistical comparisons not applicable to ANOVA tests were carried out using an unpaired, two-tailed Student's *t* test.

2.5 Transcriptomics

Total RNA from six rat livers each from vehicle control, Low PFNA + Mix, and Mid PFNA ± Mix groups were separately converted into labeled cRNA and applied to the One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) version 6.5 (Agilent Technologies, Santa Clara, CA). Labeled cRNA from each rat was hybridized to Agilent Whole Rat Genome Oligo Microarrays (G4122F) for 17 h at 65 °C. The hybridized microarrays were scanned using an Agilent DNA Microarray Scanner and evaluated using the Feature Extraction software version 10.7.3.1 according to protocol GE1_107_Sep09 (Agilent Technologies) to generate feature extraction files for further analysis. Reads were quality controlled by the software prior to release of the data. Arrays that did not pass quality control were removed from the dataset. Based on the quality control reports, two of the six microarrays from the Mid PFNA + Mix group were excluded from further analysis. The remaining arrays, six from each of control, Low PFNA + Mix, and Mid PFNA and the remaining four from Mid PFNA + Mix were found to be of high quality.

Extracted data were analyzed using the limma software package (Smyth 2004, 2005) in R (R Core Team 2012). Data were background corrected using the 'normexp' method (Ritchie et al. 2007) and normalized between arrays using quantile normalization (Smyth and Speed 2003) prior to statistical analyses. Within-array replicate probes were replaced with the average expression level. To identify treatment-specific gene effects, we fitted a linear model for each gene and applied empirical Bayes statistics (Smyth 2004) for each relevant two-group comparison. The FDR was controlled using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). Reported *p*-values for the significantly differentially expressed genes from the transcriptomics analysis were all adjusted, and p-values ≤ 0.05 were considered statistically significant.

2.6 Pathway analysis

Transcription data were analyzed with QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). IPA was used to map significantly differentially expressed gene probes to genes, and expression values were used for prediction of the involvement of the differentially expressed genes in functional networks, pathways, and diseases, and for graphical representations. Using the Fisher's Exact Test, we analyzed

the overlap between the differentially expressed genes in our dataset and genes known to be involved in disease networks, pathways, and diseases available in the Ingenuity Knowledgebase. p-values ≤ 0.05 were considered statistically significant.

3 Results and discussion

3.1 Rats exposed to low/medium levels of a 14-chemical Mix, PFNA, or both displayed no macroscopic phenotypes

Our main objective was to determine if exposure to low doses of PFNA with or without a background exposure to a low-dose chemical mixture (Mix) can have undesirable effects on the metabolome and transcriptome of animals otherwise displaying little to no phenotypic abnormalities. For this, we chose to analyze blood and liver samples from male rats that had been exposed to various levels of chemicals for a period of 14 days during juvenile age. This in vivo experiment has been described previously with a focus on low dose hormonal changes and pathological findings at high dose level (Hadrup et al. 2015). In the initial assessment of the animals, exposure to high levels of PFNA, with or without Mix, indicated hepatic steatosis of the liver; a state of retention of lipids in the liver. Animals exposed to lower levels of PFNA ± Mix, which encompasses the dose-groups included in this study, showed no obvious morphological phenotypes. However, significant plasma levels of PFNA after the exposure period were measured to be 1.1 and 30 μg/ml for Low PFNA + Mix and Mid PFNA + Mix, respectively. In animals exposed to Low-, and Mid PFNA only, plasma concentrations were 0.4 and 40 μg/ml, respectively. The former is in the range of, or up to six times the human high-end combined exposure to PFOA, PFNA and PFOS (67.6–824 ng/ml) (Emmett et al. 2006; Lau et al. 2007; NHANES 2013; Hadrup et al. 2015). Thus, the addition of Mix significantly increased the plasma levels of PFNA (2.8-fold for Low PFNA), indicating altered ADME (absorption, distribution, metabolism, excretion) properties of PFNA when Mix is co-administered.

The dose of Mix was calculated from a high-end exposure level in the European population (Christiansen et al. 2012) and corrected for body surface area of rats compared to humans (Hadrup et al. 2015). Surprisingly, none of the compounds in Mix, or their corresponding metabolites, were found when extracted ion chromatograms were created based on their accurate mass. This could be due to rapid metabolism of the compounds or that the levels were below the limit of detection in the LC–MS analysis. Nevertheless, as clear effects of PFNA levels were observed when co-administered with Mix, we chose to include all the different combinations of low- to mid-range exposure groups in our analyses aimed at discerning more subtle molecular phenotypes.

3.2 The blood plasma metabolome of rats exposed to low/medium levels of chemical mixtures, including PFNA, was significantly altered

In order to establish any potential effects caused by PFNA exposure with or without a Mix background, any effects caused by Mix alone was analyzed first. When compared to control animals, Mix exposure significantly affected 63 out of a total of 882 molecular features. As shown in Fig. 1, affected phospholipids were lysophosphatidylcholines such as (lyso-PC)(20:4) and lyso-PC(18:2), whereas affected neutral lipids included diacylglycerols (DG) such as DG(18:1/18:3) and DG(18:2/16:0), all depressed in serum of exposed animals. Plots of all significantly altered metabolites are shown in Supplementary Figs. 1 and 2, again with most of the affected metabolites being depressed relative to control.

Altered lipid metabolism is a common response to xenobiotic exposure (Karami-Mohajeri and Abdollahi 2011; Zhang et al. 2013; Androutsopoulos et al. 2013; Zhang et al. 2014). An initial stress-response to a xenobiotic insult can increase carbohydrate metabolism to meet a changing energy requirement, subsequently met by increased lipid and protein metabolism (Karami-Mohajeri and Abdollahi 2011). The general decrease in plasma lipid levels observed in the Mix-exposed rats could reflect this situation, particularly since the exposure lasted for a prolonged period (14 days), potentially resulting in effects such as hepatic injury. Interestingly, two of our identified lysophosphatidylcholines, lyso-PC(18:2) and lyso-PC(20:4) have both been identified as potential blood biomarkers for drug-induced hepatic phospholipidosis (Saito et al. 2014), and in both instances the response is reduced levels. Then, as drug-induced phospholipidosis has been associated with liver inflammation and fibrosis (Rigas et al. 1986; Lewis et al. 1989), it could suggest that we are observing compromised livers in our rats exposed to Mix, albeit not observed at the macroscopic level.

In animals exposed to Low PFNA only, we also observed changes to the blood metabolome, albeit different to what was observed with Mix only. Using PLS-DA of all the three fractions, we were able to separate the various exposure groups, with the lipid fraction depicted in Fig. 2. There was a clear difference between Low PFNA and Mix groups, but it was not possible to positively identify the specific metabolites responsible for this separation.

In all 30 metabolites separated Mix-exposed animals from animals exposed to Low PFNA (Supplementary Fig. 1). Mix alone altered 22 of these metabolites. For 6 out of 22 metabolites the concomitant exposure to Low PFNA

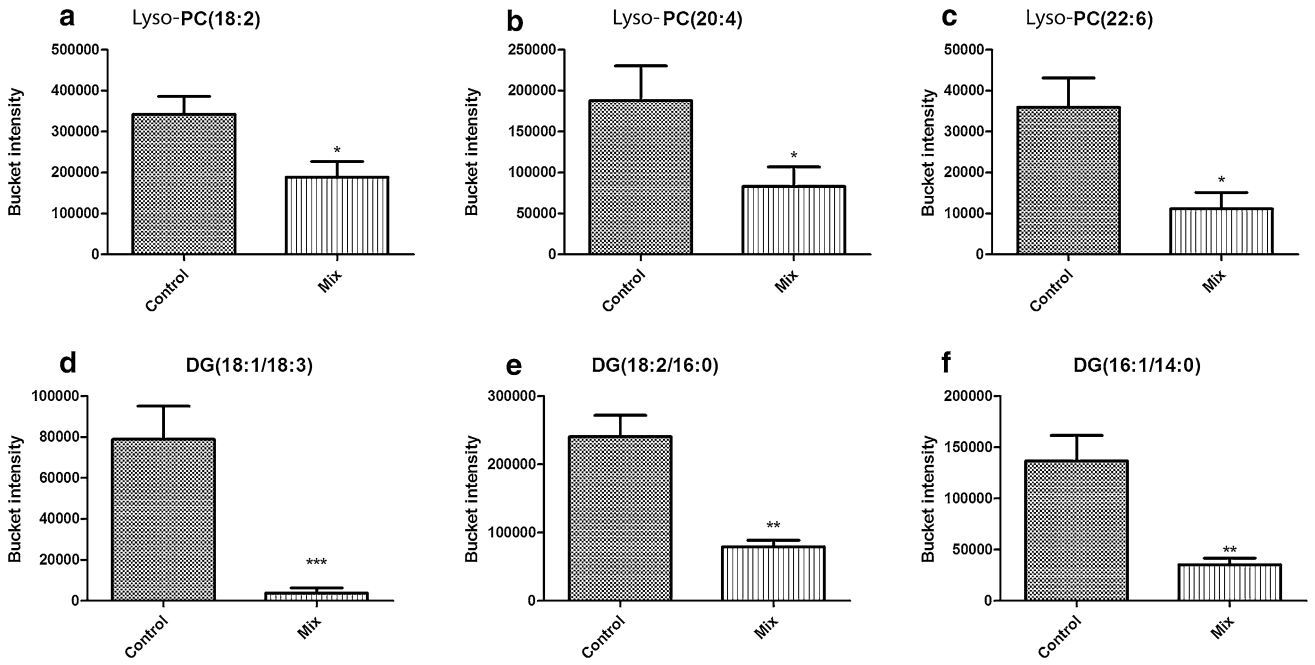

Fig. 1 The six most significantly different metabolites in the Mix group compared to the control group. In total, the levels of 63 metabolites were found to be significantly different between the two groups (*t* test). The depicted metabolites belong to two metabolite classes, diacylglycerols (DG) and lyso-phosphatidylcholines (lyso-PC)

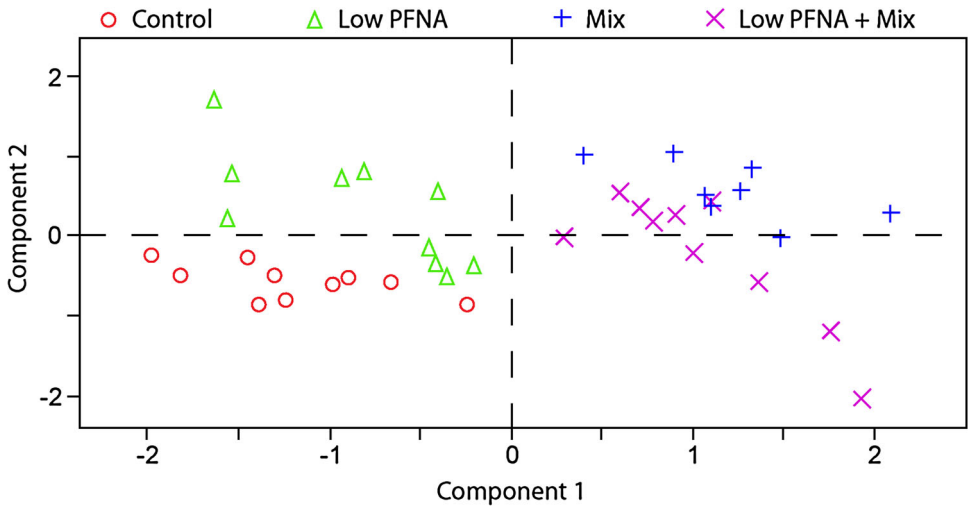

Fig. 2 PLS-DA plot of the heptane fraction correlating the four groups Control ($n = 10$), Low PFNA ($n = 10$), Low PFNA + Mix ($n = 10$) and Mix ($n = 8$). The plot illustrates that Mix accounts for a major part of the variance in the dataset

enhanced the effect on the metabolites. In the animals simultaneously exposed to Low PFNA and Mix, we observed a general depression of lipid concentrations in blood plasma, primarily diacylglycerols and the free fatty acid lignoceric acid (Fig. 3a, b). The only metabolite directly affected by Low PFNA, but not by Mix was the steroid hormone corticosterone, which was significantly elevated compared to control animals (Fig. 3c). PFNA-exposure has previously been shown to elevate plasma levels of both adrenocorticotropic hormone and cortisol in mice, though only at the highest dose of 5 mg/kg/day (Fang et al. 2008). Although it is not clear why adrenocorticotropic hormone, and subsequently glucocorticoids, are elevated by PFNA-exposure, it could be an inflammatory response, as PFNA can induce immunotoxic responses (Fang et al. 2008), and elevated endogenous glucocorticoid levels can subsequently suppress the innate immune response (Liberman et al. 2007). None of the metabolites that were altered by Mix and not by Low PFNA + Mix were identified.

We next performed the same analyses on animals having been exposed to Mid PFNA doses with or without Mix. Within these groups, we identified 24 metabolites with

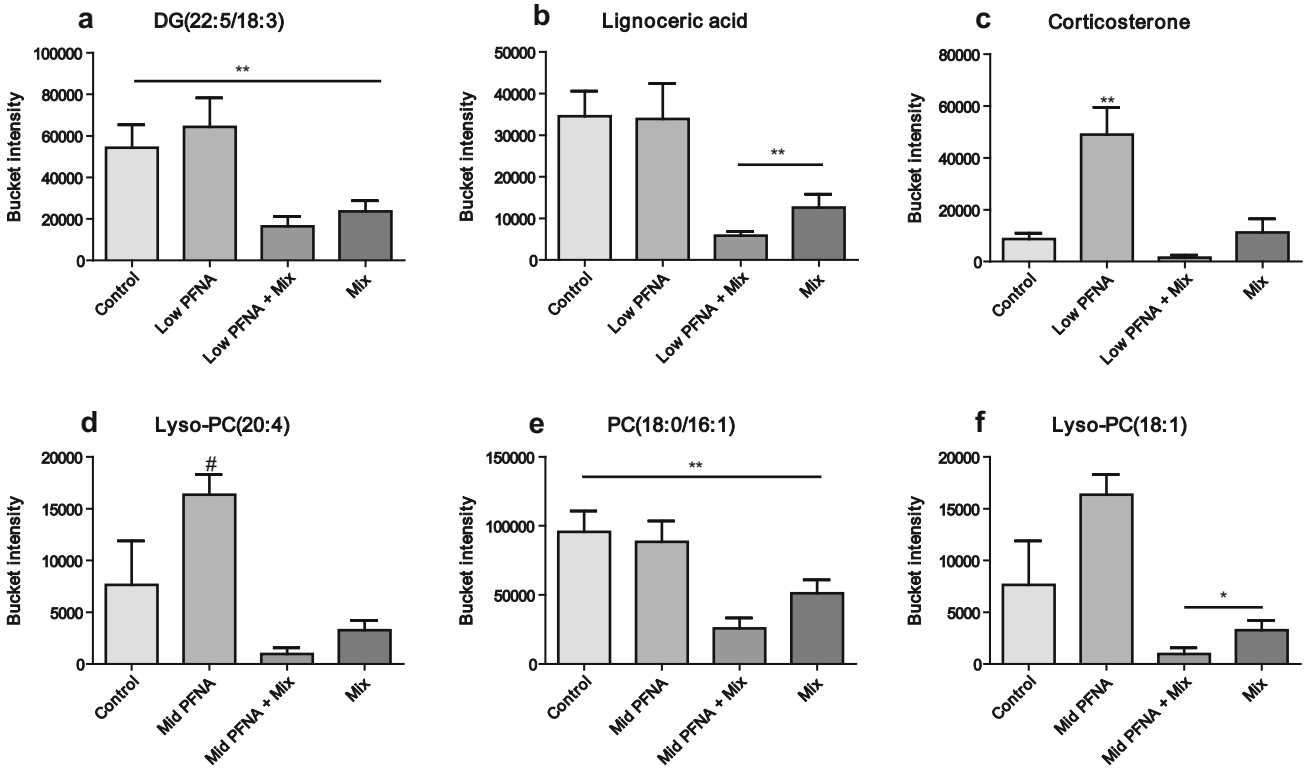

Fig. 3 Representative metabolite changes in the Low PFNA + Mix group. The *plotted* metabolites belong to two metabolite classes; PC (phosphatidylcholine) and DG (diacylglycerol). Metabolite levels were analyzed statistically for each group by comparison to control (*t* test). For corticosterone (**c**) and lyso-PC(20:4) (**d**), PFNA alone caused an increased metabolite level, whereas the combination of Mix and PFNA generally caused a decreased metabolite level (DG(22:5/18:3 (**a**) and PC(18:0/16:1) (**e**)). For lignoceric acid (**b**) and lyso-PC(18:1) (**f**) the effect by Mix + PFNA is stronger than the effect of Mix or PFNA alone. $^{\#}p$-value of 0.053

significantly changed plasma concentrations, out of which 17 were altered by Mix alone (Supplementary Fig. 2). Three representative metabolites that were significantly changed between groups are shown in Fig. 3d–f. As shown, there was an increase in the plasma level of lyso-PC(20:4) in Mid PFNA exposed animals, but not in those exposed by Mix alone. Exposure to Mix depressed plasma levels of the diacylated phospholipids PC(18:0/16:1), which was further depressed by the simultaneous exposure to PFNA despite Mid PFNA exposure alone not affecting levels of these metabolites (Fig. 3e).

To further investigate the contribution of Mix towards the PFNA-induced effects, we compared the significant metabolites resulting from statistical analysis of Mix versus control (*t* test) and PFNA + Mix versus control (ANOVA). We observed that 24 metabolites were affected by all treatments, out of which 12 were positively identified (Supplementary Fig. 3). These 12 metabolites comprised neutral short-chained lipids such as monoacylglycerols and phospholipids. Mix alone affected the diacylglycerols, whereas the PFNA + Mix groups affected the diacylated phospholipids. Surprisingly, lyso-PC(20:4) and lyso-PC(18:1) were depressed by Mix exposure, elevated by Mid PFNA exposure, but depressed beyond Mix alone when simultaneously exposed to Mid PFNA and Mix (Fig. 3d, f). Similarly, although not elevated by Mid PFNA exposure, blood plasma levels of lyso-PC(18:0) and lyso-PC(16:1) appeared to be depressed more when exposed to Mid PFNA + Mix than when exposed to Mix alone (Fig. 3e). It is difficult to discern a putative mechanism behind these observations, but it likely reflects the complex regulatory mechanisms underpinning organismal homeostasis, highlighting the importance of employing systems approaches when analyzing effects caused by mixtures of compounds. However, in some ways these metabolite changes are similar to those associated with metabolic syndrome.

Over the last decades, there has been a worldwide increase in metabolic syndrome (World Health Organization 2000), carrying with it an increased risk of developing diabetes and cardiovascular disease. Some studies have shown decreased phospholipid levels in blood plasma of diabetics (Wang et al. 2005; Liu et al. 2013) and an abnormal ratio between lyso-PC and PC levels, which may indicate a systemic change in relation to diabetes (Altmaier et al. 2008). PCs are involved in the biosynthesis of multiple compounds and a decrease in PC levels can reflect increased biosynthesis of lipids such as triacylglycerols

(Lagace and Ridgway 2013). Thus, the changes we observed in the metabolome in rats following exposure to Mix alone and PFNA + Mix were comparable to the differences observed in patients with metabolic syndrome (Ferrannini et al. 2013). Also, lyso-PCs have been shown to be elevated in metabolic syndrome patients (Chen et al. 2011).We observed elevated lyso-PCs for Mid PFNA exposure, but not for the other exposure groups. This could suggest that Mix, or components therein, protects against some of the effects caused by PFNA. However, a more likely scenario is that Mix exposure causes systemic changes that lead to a metabolic response in order to maintain tissue/serum homeostasis or to a suppressed immune response, which subsequently leads to differing responses to PFNA.

The effects observed on lipid homeostasis following exposure to Mix with or without PFNA may be attributed to the presence of glabridin, a naturally occurring isoflavonoid from licorice that has been shown to inhibit the activity of CYP2C9 in the liver (Kent et al. 2002). Studies have shown that CYP2C9 is involved in the regulation of lipid metabolism in the body (Kent et al. 2002; Kirchheiner et al. 2003). Furthermore, people carrying a polymorphism in CYP2C9 have a tendency towards having lower levels of the lipid carriers LDL and HDL (Kirchheiner et al. 2003). A decrease in LDL and HDL may result in decreased levels of plasma lipids, including cholesterol and cholesterol derivatives, as these are carrying lipids in the blood stream (Kent et al. 2002). It could also be attributed to PFNA inhibiting the activity of CYP2C9 similarly to glabridin (U. S. Environmental Protection Agency 2014).

3.3 The liver transcriptome of rats was affected by exposure to chemical mixtures, including PFNA

For global gene expression analyses, we selected to study the livers of animals exposed to Low PFNA + Mix and Mid PFNA with or without Mix relative to control, as we expected little changes in the Mix alone group. As evident in Supplementary Table 3, we observed no statistically significant ($p \leq 0.05$) changes in transcript levels of any gene in livers from Low PFNA + Mix exposed rats. Further, few genes in these groups displayed significant fold-change differences in transcript abundance, with only three genes displaying more than 1.5-fold difference: *Cyp4a11* 1.6-fold, *Cyp2b6* 2.0-fold and *Acot1* 2.1-fold. Allowing for an adjusted p-value ≤ 0.1 without a fold-change cut-off value, 31 genes could be identified as differentially expressed between the exposed and control rat livers, and are included in Table 2, which lists differentially expressed genes related to energy homeostasis. The remaining genes are listed in Supplementary Table 3.

Next we analyzed the transcriptome of livers obtained from rats exposed to Mid PFNA ± Mix. Here, only annotated genes contained in the IPA database were included when compiling lists of differentially expressed genes, thus excluding most expressed sequence tags and genes without adequate literature-based information for robust downstream analysis. At the statistically significant level of $p \leq 0.05$, 203 genes from the PFNA + Mix group and 182 genes from the PFNA only group were annotated as differentially expressed (Supplementary Table 2). Of these, the majority showed an upregulation in the exposed groups as compared to controls.

Based on functional annotation of genes dysregulated in the PFNA ± Mix dosing groups, fatty acid metabolism was the main biochemical function affected (Table 2). For instance, upregulated genes such as *Crot*, *Crat*, *Acox1*, *Ehhadh*, *Hadha*, *Hadhb*, *Decr2*, *Eci2*, *Ech1*, are all involved in peroxisomal fatty acid β-oxidation, whereas *Cpt2*, *Slc25a20*, *Acad11*, *Acadl*, *Acadm*, *Acads*, *Acadvl* are all involved in mitochondrial β-oxidation. Genes associated with lipid transport, fatty acid activation, and peroxisomal transport (*Apoa2*, *Abcd3*, *Cd36*, *Slc27a2*) were also upregulated. Amongst the downregulated genes was Fatty acid binding protein 5 (*Fabp5*). Similar effects on lipid homeostasis have been shown in other studies (Guruge et al. 2006; Rosen et al. 2007; Fang et al. 2012a, b, c). Here, the effects on lipid metabolism seem to be mainly driven by PFNA exposure, as the effects are comparable between animals exposed to Mid PFNA only and Mid PFNA + Mix.

With regards to aerobic respiration, a different transcriptional profile was observed for Mid PFNA + Mix compared to Mid PFNA alone (Table 2). For instance, the citric acid cycle enzymes, aconitase 2 (*Aco2*), isocitrate dehydrogenase 3 (NAD+) beta (*Idh3b*), and succinate-CoA ligase (*Sucla2* and *Suclg1*) were significantly upregulated by Mid PFNA, but not by Mid PFNA + Mix. Only *Aco2* and *Suclg1* were upregulated in both groups relative to control. Similarly, for enzymes in the electron transport chain (*Ndufa10*, *Ndufab1*, *Ndufs3* (complex I), and *Sdhb* (complex II)) only *Ndufa10* was significantly upregulated in both groups. The other enzymes were only upregulated in the Mid PFNA group. Finally, expression of genes encoding enzymes involved in glucose metabolism were mainly disrupted by Mid PFNA + Mix, except for a few genes such as phosphofructokinase (*Pfkm*) that were only significant with Mid PFNA and carbonic anhydrase VII (*Ca7*) and STEAP family member 4 (*Steap4*), which were differentially expressed by both groups. Thus, the transcriptional profiles of Mid PFNA ± Mix are relatively similar with only subtle differences observed based on z-scores and p-values. Notably, a statistical comparison between Mid PFNA and Mid PFNA + Mix did not yield

Table 2 Genes related to energy homeostasis that were altered by Mid PFNA + Mix

Symbol	Entrez ID	Entrez gene name	Log_2(fold change)			Biological process
			Low PFNA + Mix	Mid PFNA	Mid PFNA + Mix	
Aco1	50655	Aconitase 1, soluble	0.12	*0.337*	**0.544**	Aerobic metabolism
Aco2	79250	Aconitase 2, mitochondrial	−0.065	**0.428**	**0.448**	Aerobic metabolism
Cox11	690300	Cytochrome c oxidase assembly homolog 11 (yeast)	0.24	**0.593**	0.235	Aerobic metabolism
Idh3b	94173	Isocitrate dehydrogenase 3 (NAD +) beta	0.003	**0.325**	0.247	Aerobic metabolism
Ndufa10	678759|316632	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42 kDa	0.042	**0.3**	**0.275**	Aerobic metabolism
Ndufab1	293453	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8 kDa	0.051	**0.269**	0.116	Aerobic metabolism
Ndufs3	295923	NADH dehydrogenase (ubiquinone) Fe–S protein 3, 30 kDa (NADH-coenzyme Q reductase)	0.085	**0.336**	*0.28*	Aerobic metabolism
Sdhb	298596	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	0.043	**0.244**	0.17	Aerobic metabolism
Sucla2	361071	Succinate-CoA ligase, ADP-forming, beta subunit	0.145	**0.43**	0.357	Aerobic metabolism
Suclg1	114597	succinate-CoA ligase, alpha subunit	0.024	**0.275**	**0.284**	Aerobic metabolism
Acaa1	501072|24157	Acetyl-CoA acyltransferase 1	0.113	**1.008**	**0.985**	Lipid metabolism
Acaa2	170465	Acetyl-CoA acyltransferase 2	0.394	0.556	**0.969**	Lipid metabolism
Acot1	314304	Acyl-CoA thioesterase 1	0.386	0.984	**1.427**	Lipid metabolism
Acot12	170570	Acyl-CoA thioesterase 12	0.245	**0.557**	**0.666**	Lipid metabolism
Acot2	192272	Acyl-CoA thioesterase 2	0.241	**1.312**	**1.497**	Lipid metabolism
Acot4	681337	Acyl-CoA thioesterase 4	0.264	1.115	**1.585**	Lipid metabolism
Acot7	26759	Acyl-CoA thioesterase 7	0.019	**0.431**	**0.593**	Lipid metabolism
Aldh1a1	24188	Aldehyde dehydrogenase 1 family, member A1	0.377	**2.077**	**2.523**	Lipid metabolism
Cyp2b6	361523|24300	Cytochrome P450, family 2, subfamily B, polypeptide 6	*0.971*	**2.12**	**2.561**	Lipid metabolism
Cyp2c19	293989	Cytochrome P450, family 2, subfamily C, polypeptide 19	0.128	**0.686**	**0.756**	Lipid metabolism
Cyp2j2	65210	Cytochrome P450, family 2, subfamily J, polypeptide 2	0.237	**1.018**	**1.187**	Lipid metabolism
Cyp4a11	50549	Cytochrome P450, family 4, subfamily A, polypeptide 11	0.643	**2.419**	**2.568**	Lipid metabolism
Cyp4a14	298423|24306	Cytochrome P450, family 4, subfamily a, polypeptide 14	0.333	**1.201**	**1.335**	Lipid metabolism
Decr1	117543	2,4-Dienoyl CoA reductase 1, mitochondrial	0.234	**1.364**	**1.344**	Lipid metabolism
Ephx2	65030	Epoxide hydrolase 2, cytoplasmic	0.243	**1.201**	**1.267**	Lipid metabolism
Gcdh	364975	Glutaryl-CoA dehydrogenase	0.175	**0.576**	**0.638**	Lipid metabolism
Hadh	113965	Hydroxyacyl-CoA dehydrogenase	0.056	**0.418**	**0.4**	Lipid metabolism
Hadha	170670	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	0.144	**0.659**	**0.746**	Lipid metabolism
Hadhb	171155	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	0.194	**0.8**	**0.817**	Lipid metabolism

Table 2 continued

Symbol	Entrez ID	Entrez gene name	Log_2(fold change)			Biological process
			Low PFNA + Mix	Mid PFNA	Mid PFNA + Mix	
Hsd11b1	25116	Hydroxysteroid (11-beta) dehydrogenase 1	−0.261	−0.446	**−1.082**	Lipid metabolism
Immt	312444	Inner membrane protein, mitochondrial	−0.011	**0.345**	**0.376**	Lipid metabolism
Mlycd	85239	Malonyl-CoA decarboxylase	0.093	**0.316**	**0.325**	Lipid metabolism
Ncam1	24586	Neural cell adhesion molecule 1	−0.163	−0.05	**−0.27**	Lipid metabolism
Phgdh	58835	Phosphoglycerate dehydrogenase	−0.308	**−1.271**	**−0.976**	Lipid metabolism
Plin5	501283	Perilipin 5	0.198	**0.595**	**0.61**	Lipid metabolism
Ptk2b	50646	Protein tyrosine kinase 2 beta	0.175	**0.607**	**0.47**	Lipid metabolism
Sirt4	304539	Sirtuin 4	0.162	0.291	**0.461**	Lipid metabolism
Acad11	315973	Acyl-CoA dehydrogenase family, member 11	0.115	**0.62**	**0.656**	Lipid metabolism—mitochondrial b-oxidation
Acadl	25287	Acyl-CoA dehydrogenase, long chain	0.05	**0.442**	**0.419**	Lipid metabolism—mitochondrial b-oxidation
Acadm	24158	Acyl-CoA dehydrogenase, C-4 to C-12 straight chain	0.15	**0.47**	**0.47**	Lipid metabolism—mitochondrial b-oxidation
Acads	64304	Acyl-CoA dehydrogenase, C-2 to C-3 short chain	0.056	**0.326**	**0.416**	Lipid metabolism—mitochondrial b-oxidation
Acadvl	25363	Acyl-CoA dehydrogenase, very long chain	0.047	**0.413**	**0.482**	Lipid metabolism—mitochondrial b-oxidation
Cpt2	25413	Carnitine palmitoyltransferase 2	0.163	**0.565**	**0.786**	Lipid metabolism—mitochondrial b-oxidation
Slc25a20	117035	Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	0.098	**0.668**	**0.692**	Lipid metabolism—mitochondrial b-oxidation
Acox1	50681	Acyl-CoA oxidase 1, palmitoyl	0.074	**0.601**	**0.745**	Lipid metabolism—proxisomal b-oxidation
Crat	311849	Carnitine *O*-acetyltransferase	0.372	**1.55**	**1.71**	Lipid metabolism—proxisomal b-oxidation
Crot	83842	Carnitine *O*-octanoyltransferase	0.19	**1.115**	**1.219**	Lipid metabolism—proxisomal b-oxidation
Decr2	64461	2,4-Dienoyl CoA reductase 2, peroxisomal	0.275	**0.831**	**0.944**	Lipid metabolism—proxisomal b-oxidation
Ech1	64526	Enoyl CoA hydratase 1, peroxisomal	0.459	**1.503**	**1.856**	Lipid metabolism—proxisomal b-oxidation
Eci2	291075	Enoyl-CoA delta isomerase 2	0.117	**0.577**	**0.548**	Lipid metabolism—proxisomal b-oxidation
Ehhadh	171142	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	0.184	**1.702**	**1.86**	Lipid metabolism—proxisomal b-oxidation
Hsd17b4	79244	Hydroxysteroid (17-beta) dehydrogenase 4	0.113	*0.295*	0.278	Lipid metabolism—proxisomal b-oxidation
Apoa2	25649	Apolipoprotein A-II	0.321	**0.504**	*0.474*	Lipid metabolism—transport
Abcd3	25270	ATP-binding cassette, sub-family D (ALD), member 3	0.093	**0.729**	**0.685**	Lipid metabolism—transport
Cd36	29184	CD36 molecule (thrombospondin receptor)	0.158	**1.276**	**1.867**	Lipid metabolism—transport
Fabp5	140868	Lipid binding protein 5 (psoriasis-associated)	−0.385	**−1.392**	**−1.508**	Lipid metabolism—transport
Slc27a2	65192	Solute carrier family 27 (Lipid transporter), member 2	0.215	**0.543**	**0.57**	Lipid metabolism—transport
Adrb3	25645	Adrenoceptor beta 3	−0.279	−0.134	**−0.366**	Glucose metabolism disorders
Ca1	310218	Carbonic anhydrase I	−0.003	*−0.78*	**−1.132**	Glucose metabolism disorders
Ca4	29242	Carbonic anhydrase IV	*−0.266*	−0.009	**−0.236**	Glucose metabolism disorders
Ca7	291819	Carbonic anhydrase VII	–0.06	**0.817**	**0.643**	Glucose metabolism disorders
Calcr	116506	Calcitonin receptor	0.225	0.114	**0.392**	Glucose metabolism disorders

Table 2 continued

Symbol	Entrez ID	Entrez gene name	Log_2(fold change)			Biological process
			Low PFNA + Mix	Mid PFNA	Mid PFNA + Mix	
Dnajc3	63880	DnaJ (Hsp40) homolog, subfamily C, member 3	−0.113	**−0.279**	**−0.362**	Glucose metabolism disorders
Fbp2	114508	Fructose-1,6-bisphosphatase 2	0.272	*0.642*	**1.081**	Glucose metabolism disorders
Hdc	24443	Histidine decarboxylase	0.267	1.03	**1.309**	Glucose metabolism disorders
Hipk1	365895	Homeodomain interacting protein kinase 1	0.189	0.011	**0.296**	Glucose metabolism disorders
Lef1	161452	Lymphoid enhancer-binding factor 1	0.1	0.36	**0.573**	Glucose metabolism disorders
Mgat2	94273	Mannosyl (alpha-1,6-)-glycoprotein beta-1,2-*N*-acetylglucosaminyltransferase	−0.135	*−0.436*	**−0.544**	Glucose metabolism disorders
Pfkm	65152	Phosphofructokinase, muscle	−0.388	**−0.487**	−0.35	Glucose metabolism disorders
Prox1	305066	Prospero homeobox 1	−0.09	−0.114	**−0.336**	Glucose metabolism disorders
Psmc3	29677	Proteasome (prosome, macropain) 26S subunit, ATPase, 3	0.102	*0.287*	**0.326**	Glucose metabolism disorders
Psmd8	292766	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	0.103	0.179	**0.238**	Glucose metabolism disorders
Ramp1	58965	Receptor (G protein-coupled) activity modifying protein 1	−0.133	−0.213	**−0.299**	Glucose metabolism disorders
Rt1-Ba	309621	Major histocompatibility complex, class II, DQ alpha 1	*−0.244*	−0.139	**−0.302**	Glucose metabolism disorders
Steap4	499991	STEAP family member 4	−0.15	**−0.332**	**−0.404**	Glucose metabolism disorders
Hmgcs2	24450	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	0.118	**0.469**	**0.471**	Ketogenesis
Pex11a	85249	Peroxisomal biogenesis factor 11 alpha	0.209	**1.368**	**1.387**	Peroxisome biogenesis
Pex16	311203	Peroxisomal biogenesis factor 16	0.208	**0.405**	**0.439**	Peroxisome biogenesis
Pex5	312703	Peroxisomal biogenesis factor 5	0.077	**0.326**	*0.284*	Peroxisome biogenesis
Dhrs4	266686	Dehydrogenase/reductase (SDR family) member 4	0.005	**0.363**	0.294	Retinol metabolism

Roman non-significant, *italic* $p = 0.05$–0.1, *bold* $p < 0.05$

Table 3 Upstream regulators predicted to regulate effects caused by Mid-PFNA ± Mix

Upstream regulator	Mid PFNA + Mix		Mid PFNA		Genes
	z-score	*p*-value	*z*-score	*p*-value	
PPARα Peroxisome proliferator-activated receptor α	5.3	2e–24	5.1	5e–28	Abcd3, Acaa1, Acaa2[a], Acadl, Acadm, Acads, Acadvl, Acat1, Acot1[a], Acot2, Acox1, Adtrp, Cd36, Cpt2, Crot, Cyp2b6, Cyp4a11, Cyp4a14, Decr1, Decr2, Ech1, Eci2, Ehhadh, Fabp5, Gpd1, Hadh, Hadha, Hadhb, Rt1-Ba[a], Hmgcs2, Mlycd, Pex11a, Plin5, Slc25a20, Slc27a2, Vnn1, Aadac[b], Apoa2[b], Cfh[b], Ftcd[b], H2afz[b]
KLF15 Krüppel-like factor 5	3.2	3e–18	3.0	5e–17	Acadl, Acadm, Acadvl, Acot1[a], Acox1, Cd36, Cpt2, Decr1, Ehhadh, Fabp5, Hadha, Hadhb, Mlycd, Slc25a20
ACOX1 ↑Peroxisomal acyl-coenzyme A oxidase 1	−2.4	5e–15	−2.474	3e–13	Abcd3, Acaa1, Acadl, Acadm, Acadvl, Acot2, Acox1, Aig1, Cd36, Crat, Cyp4a11, Cyp4a14, Ehhadh, Rt1-Ba[a], Hsd11b1[a], Pex11a, Slc27a2, Tnfrsf10a[a], Hspa5[b]
PPARγ Peroxisome proliferator-activated receptor α	3.9	8e–14	3.3	1e–13	Acaa1, Acaa2[a], Acadl, Acadm, Acads, Acox1, Adrb3[a], Cd36, Cpt2, Crat, Cyp4a11, Cyp4a14, Ehhadh, Fabp5, Fbp2[a], Gpd1, Hadha, Hadhb, Hmgcs2, Mlycd, Pepd, Pex11a, Plin5, Slc25a20, Vnn1, Apoa2[b], Sdc1[b]
EHHADH ↑Enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase	−2.8	1e–12	−2.8	6e–13	Abcd3, Acaa1, Acot2, Acox1, Cd36, Cyp4a11, Cyp4a14, Pex11a
HSD17B4 ↑Peroxisomal multifunctional enzyme type 2	−3.0	1e–12	−2.8	4e–11	Abcd3, Acaa1, Acot2, Acox1, Cd36, Cyp4a11, Cyp4a14, Hoxd13[a], Pex11a

An arrow indicating up- or downregulation marks upstream regulators that are differentially expressed genes. Negative *z*-scores indicate that the upstream regulator is inactivated, whereas positive *z*-scores indicate activation

[a] DEG only present in Mid PFNA + Mix

[b] DEG only present in Mid PFNA

any differentially expressed genes, hence further studies with larger sample sizes are required to verify some of the subtle differences indicated by this study.

The IPA has a function to predict the activity of potential upstream regulators based on differentially expressed genes. Using our dataset, we could predict an increased activity of several transcription factors and other central proteins in livers from exposed animals (Table 3). Downstream of the differentially expressed genes, IPA predicts regulatory effects such as diseases and functions likely to be perturbed due to the changes in gene expression. As illustrated in Fig. 4, activation of the upstream regulators PPARα, PPARγ, PPARδ, RXRα, PPARgc1a, and MED1 results in altered gene expression (Table 2) that subsequently leads to increased oxidation of lipids, in particular fatty acids. This, in turn, protects against accumulation of lipids in the liver and therefore also against hepatic steatosis. This is consistent with our observations in livers from rats having been exposed to Low- and Mid PFNA doses, with no indications of lipid accumulation, increased cell size, or compromised cell borders (Hadrup et al. 2015). However, as exposure to high PFNA doses causes adverse effects such as steatosis (Hadrup et al. 2015), it can be speculated that livers from at least the Mid PFNA exposed rats could become affected over time. The fact that hepatic steatosis was evident at the high PFNA dose (5 mg/kg/day) and that signs of protection against hepatic steatosis was observed at the mid PFNA dose (0.25 mg/kg/day) illustrates the problem of extrapolating from toxicological observations at higher doses to effects at lower doses, which is common practice in risk assessment of chemicals.

PPARs are known regulators of fatty acid β-oxidation (Kanehisa and Goto 2000), and increased β-oxidation results in decreased plasma lipid concentration (Lau 2012). Then, as perfluorinated alkyl acids are known PPAR-activators (Vanden Heuvel et al. 2006; Lau 2012; U. S. Environmental Protection Agency 2014), effects observed in livers from the PFNA groups could, at least in part, be explained by this regulatory pathway. Further, the Mix contains two phthalates, DBP and DEHP, which are both known PPAR activators (Desvergne et al. 2009), potentially adding to the effect in the PFNA + Mix groups. In fact, additional compounds in the Mix such as butylparaben, linuron, and vinclozolin, can stimulate PPAR-dependent transcription (U. S. Environmental Protection Agency 2014), further adding to the overall effect. However, at the doses used in our study, the contribution of Mix

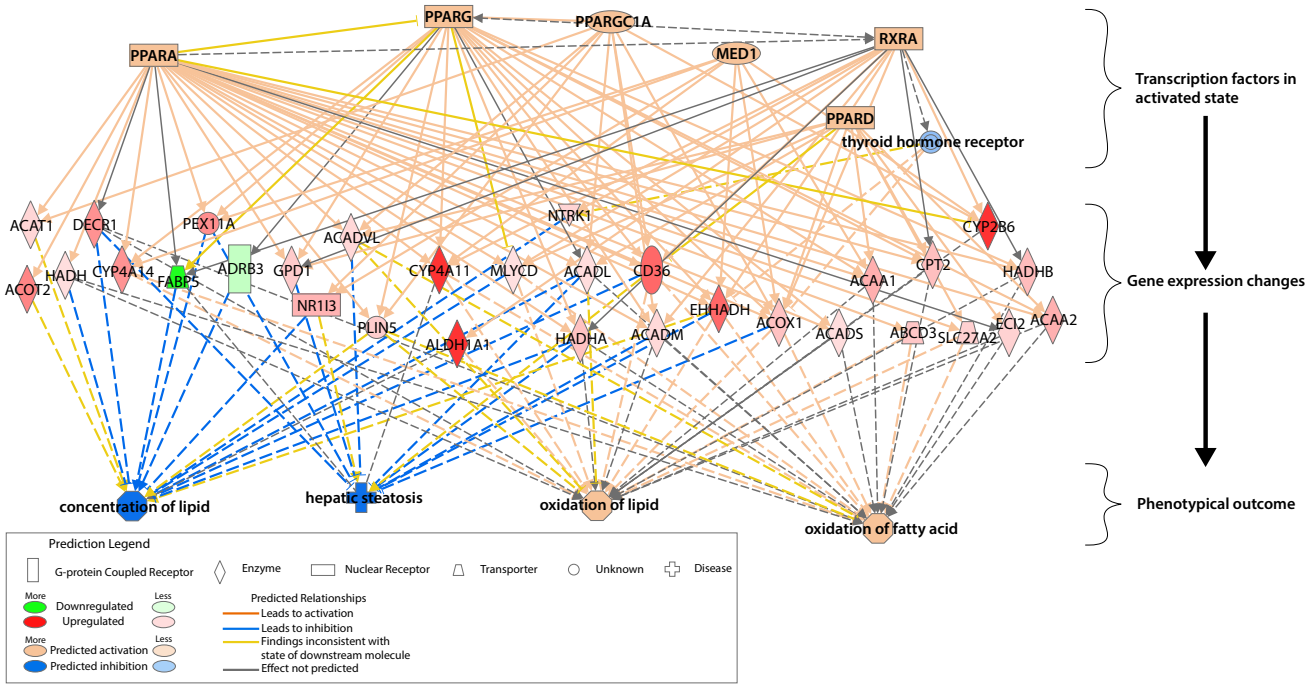

Fig. 4 Illustration of a putative hepatic signaling network, from PPAR (and other transcriptional regulators) activation through regulation of gene expression to ultimately dictate phenotypic outcomes. *Orange* indicates predicted activation and *blue* indicates predicted inhibition. Shades of *red* and *green* indicate level of increased and decreased gene expression, respectively. The network was generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) (Color figure online)

towards PPAR activation is likely minor since (i) we observed very small differences in transcript levels between the Mid PFNA and Mid PFNA + Mix groups, and (ii) we did not observe any significant changes in gene expression in animals exposed to Low PFNA + Mix.

A summary of the functions of the Mid PFNA ± Mix is given in Supplementary Table 4, together with genes that displayed highest fold-change difference in transcript levels between Mid PFNA + Mix and Mid PFNA alone. As discussed, altered CYP2C9 activity might play a role in the changed plasma lipid concentrations. However, our data do not suggest downregulation of the rat CYP2C9 homologue, *Cyp2c11*. On the other hand, the HDL-constituent, apolipoprotein A-II (encoded by *Apoa2*) is upregulated by PFNA but not by PFNA + Mix. The effects observed for Mix and PFNA + Mix might therefore be caused by a combination of decreased CYP2C9 activity and PPAR activation.

4 Concluding remarks

The characterization of potential toxic effects caused by prolonged exposure to various compounds can be a challenge. When a large number of compounds are present at the same time, as in humans, the challenge is even greater. In an effort to better understand the complex interaction between multiple exogenous compounds and the living organism, we profiled the plasma metabolome and the liver transcriptome in rats after exposure to a low dose of bioavailable chemicals. We observed that a mixture of 14 chemicals at high-end human exposure levels (Mix), low- to mid-range doses of PFNA, or a combination of the two, had the potential to alter the blood plasma levels of diacylglycerols, PCs, and cholesterol derivatives, mimicking some of the features of metabolic syndrome. These effects can also suggest an inflammatory response, but in either case warrant further investigations. We also observed more subtle changes to the liver transcriptome following Mid PFNA exposure irrespective of Mix, suggesting that the hepatic effects were driven by PFNA. In conclusion, this study demonstrates that low-dose exposure to chemical mixtures can affect the metabolome and cause disturbed lipid homeostasis, and that 'omics' approaches are powerful tools to detect smaller changes not readily observable at the macroscopic level.

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Conflict of interest Kasper Skov, Kristine Kongsbak, Niels Hadrup, Henrik Lauritz Frandsen, Terje Svingen, Jørn Smedsgaard, Karine Audouze, Aron Charles Eklund, and Anne Marie Vinggaard declare that they have no conflict of interest.

Compliance with ethical requirements All applicable institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. The animal study was approved by the Danish Animal Experiments Inspectorate. The authorization number given is 2012/561-188. The National Food Institute's in-house Animal Welfare Committee for animal care and use supervised the experiments.

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Paper IV Effect of BPA on rats and their offsprings – a metabolic profiling of low dose exposure to BPA

Manuscript in progress

Aim: Determination of low dose effects of BPA on the plasma of mother rat and offspring.

Results: The dosing of BPA affected the mother animals and offspring differently. The metabolic change of the mother animals suggests changes of the lipid metabolism with an increase level of cholesterol and a decreased level of monoacylglycerol. The plasma of male offspring showed an increased amount of phosphotidylcholine compounds.

Conclusion: Low dose effects on the rat metabolome are observed as they are exposed to Bisphenol A. The mother animals had a difference in plasma concentration of monoacylglycerol and cholesterol. The male offsprings had an increase in plasma concentration of lyso-phosphotidylcholine concentration.

Metabolic Profiling of Rats and their Offspring Exposed to Low Doses of Bisphenol A.

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Abstract

Bisphenol A (BPA) is an industrial chemical used to manufacture polycarbonate plastics, epoxy resins and thermal printings. BPA is detectable in consumer products, food, water and dust. Human exposure has been confirmed by analysis of urine, blood and tissue. Metabolomics analysis was performed analyzing plasma of rats exposed to two doses of BPA, 0.025 and 0.25 mg/kg bw/day. This concept was used to identify low dose effects on the plasma from both dams, as well as male and female offspring.

The metabolome of the dams and offspring were analyzed by a PCA plotting. The dams and the offspring located into two distinctive groups reflecting differences in age and route of exposure. Moreover, the male and female offspring had similar clustering in the PCA plot. To identify difference between control and exposed animals, PLS-DA plots of each of the three animal groups were conducted. Twenty-seven molecular features out of 347 were found to be significantly different between controls and male offspring. For lyso-phosphatidylcholine compounds a “dose response” trend was observed, while for the neutral lipids and for a metabolite with the molecular weight 359.149 there were only effects at BPA-0.25. For the dams a BPA induced increase in cholesterol in the plasma was observed.

A difference in the metabolome between male and female rats was observed. Another observeda difference was between dams administered to BPA directly and the offspring exposed through the dams (via placenta and milk in lactation period). The lyso-phospholipids was increased male offspring when exposed to BPA-0.25. In conclusion, the dams showed increased levels of cholesterol and a decreased level of monoacylglycerols while the males and the female offspring showed an effect on diacylglycerol.

Introduction

Bisphenol A (BPA) is an industrial chemical used to manufacture polycarbonate plastics, epoxy resins and thermal printings (Boucher et al. 2014). BPA is detectable in consumer products, food, water and dust (Alonso-Magdalena et al. 2008; Asahi et al. 2010; Boucher et al. 2014). Human exposure has been confirmed by analysis of urine, blood and tissue (Asahi et al. 2010). The biomarkers of BPA is the compound itself along with the major metabolite; glucuronidated BPA (Bjerregaard et al. 2007). Exposure is correlated with diseases such as diabetes and obesity (Alonso-Magdalena et al. 2008), though no specific mechanisms of action have yet been determined (Asahi et al. 2010; Boucher et al. 2014). Correlations between BPA concentration and diseases such as heart failure and diabetes have been shown (Srivastava and Godara 2013). Furthermore, BPA have been shown to be transferred from mother to child in placental cord blood (Srivastava and Godara 2013). In children chronically exposed to BPA gastrointestinal problems, reduced immune system, toxic overload and neurological disorders have been demonstrated (Srivastava and Godara 2013). The dose of the previously described studies is above the "no observed adverse effect level" (NOAEL) of 5 mg/kg bw per day. In the offspring that are also included in the current study both male and female anogenital distance was significantly decreased at birth at low doses (from 0.250 mg/kg in males and from 0.025 mg/kg in females) and above. Moreover, the incidence of nipple retention in males appeared to increase dose relatedly and the increase was statistically significant at 50 mg/kg per day (Christiansen et al. 2014). Multiple studies have suggested that even exposure to low doses of BPA during development will have effect on a range of different endpoints. Studies in rodents have reported effects of BPA at or below 50µg/kg per day on reproductive organ weights, mammary gland development and behavior (Christiansen et al. 2014; Xu and Xu 2013). These effects of BPA emphasize the need to understand the mechanism of action for BPA. The mechanism of action is not clarified and may involve several differential mechanisms of actions. It is probably not limited to the classical estrogenic mechanism (Shelby 2008) but likely also comprises several other signaling pathways (Reif et al. 2010).

In the recent years new methods have been used to analyze the effects of BPA administered at low doses. Among these methods are the "omics" methods; transcriptomics, proteomics and metabolomics. Metabolomics is the comprehensive analysis of all the metabolites in a given organism, normally compounds with a molecular weight of less than 1 kDa (Brown et al. 2005; Goodacre et al. 2004; Oliver et al. 1998). Changes in the metabolome have been found in zebrafish and rats at doses of BPA at least ten times lower than the NOAEL value of 5 mg/kg bw/day. Grasselli *et al.* show difference in the transcriptome involved in the lipid and glucose homeostasis in rat hepatoma cells (Grasselli et al. 2013). This is supported by Chen *et al.* showing changes in the lipid metabolome in rats exposed to BPA (Chen et al. 2012) and

Alongo-Magdalena who has shown that BPA induces insulin producing in the Langerhans islets (Alonso-Magdalena et al. 2008). Furthermore, Xu and Xu showed changes in the polar fraction of the metabolome when exposing rats to a low dose of 1 µg/kg bw/day for 45 days (Xu and Xu 2013).

Well above 600 articles have been published on toxic effects of BPA; however several modes of action has yet been established (Asahi et al. 2010; Reif et al. 2010). The aim of the present study was to investigate the effect of perinatal low dose exposure of BPA on the metabolome of female Wistar rats and their offspring to obtain data that may help determine a mechanism of action. Metabolomics analysis of two doses of BPA, 0.025 and 0.25 mg/kg/day, corresponding to 200 and 20 times below the no observed adverse effect level, was done. Analysis of the early sexual development such as anogenital distance and nipple retention from the same study is described elsewhere (Christiansen et al. 2014). By use of metabolomics we identified low dose effects on the plasma from both dams, as well as male and female offspring, effects that may be a first step towards identifying a mode of action.

Materials and methods

Chemicals

BPA (purity of 99.5%, CAS no. 80-05-7) was purchased from Sigma–Aldrich. Corn oil used both as negative control and as vehicle was purchased from Sigma–Aldrich. The corn oil was provided to the laboratory in glass bottles. The dosing solutions were kept in glass bottles in the dark, at room temperature, and continuously stirred during the dosing period. The levels of BPA in the solutions were verified by chemical analysis as described in Christiansen et al. (2014).

Animals and treatment

The Animal study was carried out at the DTU National Food Institute (Mørkhøj, Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given: 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

A total of 110 time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at gestation day (GD) 3 of pregnancy.

The animals were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden). The study was performed using three blocks (separated by 1 week), and all dose groups were equally represented in the blocks, i.e. the 22 time-mated rats per dose group were allocated among blocks. The dams were distributed into five dose groups (0, 0.025, 0.250, 5 or 50 mg/kg bw per day respectively). Details on study design are described in Christiansen et al. (2014).

Samples for metabolomics

On PD (pupday) 22 the dams as well as one male and one female from 10 litters per dose group (0; 0.025; 0.25 mg/kg) were weighed and decapitated, blinded with respect to exposure groups. The dams were dosed 1½-2 hours prior to section to take into account the toxicokinetics of the compound. Serum was frozen for metabolomics (see Table 1 below).

Table 1 Dams and offspring analyzed by metabolomics

	Dams	Males	females
1: Control	10	10	10
2: BPA-0.025	10	10	10
3: BPA-0.25	10	10	10

Metabolomics analysis

The applied procedure is described in (Skov et al. n.d.). The main difference to this procedure is that in this study only 40 µl of plasma was used for the analysis. Furthermore, the extraction solvents were reduced to 100 µl. In brief, phospholipids were removed using a phospholipid SPE column (Supelco, Sigma-Aldrich). The eluate was collected, dried and extracted by 100 µl heptane in order to remove the lipids. This was followed by addition of 100 µl methanol in order to extract the more polar compounds. The phospholipids were next eluted from the SPE column using 300 µl 10 % NH_4OH in methanol. The phospholipid, lipid and the polar fractions were analyzed by an HPLC system combined with a maxis qTime-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) (Skov et al. n.d.). All samples within each fraction were randomized before analysis.

Data analysis

The LC data was analyzed by Profile Analysis 2.1 (Bruker Daltonics, Bremen, Germany) using the find molecular features algorithm. The data matrix was extracted and noise was reduced by use of an R script (R Core Team 2012). This served to remove peaks that were present in < 50% of the samples among all treatment groups and at the same time had a peak intensity of ≤ 3000. The data from each group of animals were then uploaded to the online software program Metaboanalyst (Metaboanalyst.ca) (Xia et al. 2009). Metabolites that were changed in their blood concentrations were identified by doing normalization by the sum of all peaks, LOG transformation and Pareto scaling followed by ANOVA analysis in Metaboanalyst. A P>0.05 false discovery rate correction was applied to adjust for the high number of metabolites present in blood. These findings were used to select pseudo ions of interest that were further analyzed statistically as described below. Accurate mass searches MSMS patterns were used to suggest metabolites of interest. The MSMS pattern was compared with *in silico* data from lipidmaps or the Human Metabolome Database (Fahy et al. 2009; Xia et al. 2009, 2012, 2013).

Statistical analysis

The D'Agostino and Pearson omnibus normally test was used to test for normality of the data. If data were normally distributed, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was performed on plasma level of metabolites, and pathology data. Otherwise, data was LOG transformed and, if normally distributed, analyzed by ANOVA. If not normally distributed a non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparisons test was conducted. The criteria for statistical significance was $p<0.05$, $p<0.01$ and $p<0.001$ leading to marking *, ** and ***, respectively.

Determination of metabolite identities

Significantly different metabolites were identified by accurate mass search on databases such as human metabolome database (hmdb.ca) (Wishart et al. 2007), lipidmaps (lipidmaps.org) (Fahy et al. 2009), massbank (massbank.jp) and metlin (metlin.scripts.edu) (Trauger et al. 2005). If a possible metabolite was suggested the MSMS pattern was correlated with MSMS patterns from lipidmaps or HMDB databases. For some of the compounds was a standard of the compound purchased and the spectra and retention time of the standard correlated with spectra and retention time of the significantly changed metabolite.

Results

Toxicological findings

Analysis of the early sexual development in the current study showed a significantly decreased anogenital distance at birth at low doses (from 0.250 mg/kg in males and from 0.025 mg/kg in females) and above.

Moreover, the incidence of nipple retention in males appeared to increase dose relatedly and the increase was statistically significant at 50 mg/kg per day (Christiansen et al. 2014).

Metabolomics

A Partial Least square-discriminant analysis (PLS-DA) was conducted separately for dams, female and male offspring (Figure 1). The dose groups were clearly separated in the PLS analyses.

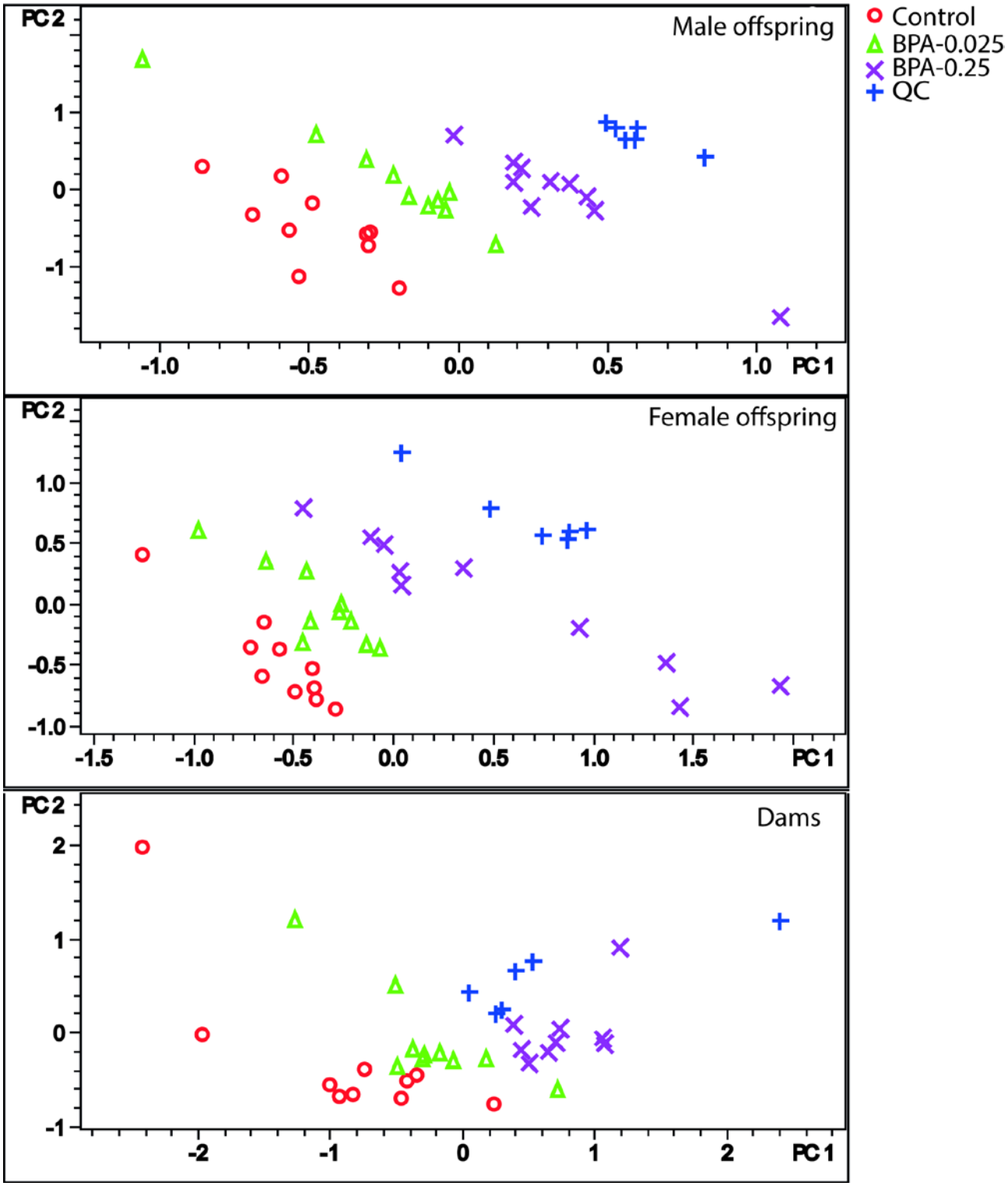

Figure 1 PLS-DA plots of the methanol fraction of male offspring, female offspring and dams. As shown on the plots are the groups and QC samples located closely together. The female and male offspring seem to have the same kind of separation while the change in the metabolites of the dams seems to be a bit more separated.

The metabolome of the dams and offspring were analyzed by a PCA plot (data not shown). The dams and the offspring located into two distinctive groups reflecting differences in age and route of exposure.

Moreover, the male and female offspring had similar clustering in the PCA plot. To identify differences between control and exposed animals, PLS-DA plots of each of the three animal groups was conducted. The location of the different treatment groups of male and female offspring, were similar on the PLS-DA plot. However, the metabolites accounting for a dose dependent difference in between all groups the metabolic change were not the same. The PLS-DA plot for the dams showed a greater change, but a closer clustering of the exposed and control animals. The significantly changed metabolites are described below.

Changes in dams:

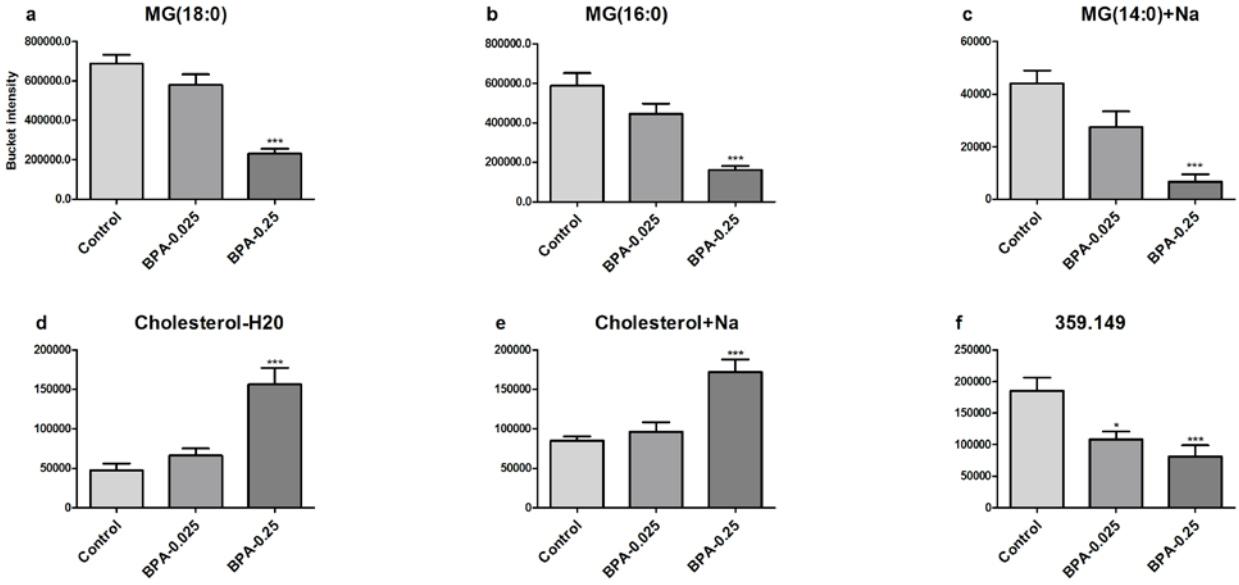

Figure 2 Metabolites significantly changed in the metabolome of the dams. The first three metabolites (a-c) are monoacylglycerols (MG) which are all down-regulated by BPA-0.25. MG(18:0) and MG(16:0) and their adducts account for 10 of the 29 molecular features found significantly changed between control and BPA exposed animals. Furthermore, cholesterol was found as a loss of water and as a Na adduct both were increase compared to control. F is a metabolite not identified. The criteria for statistical significance was $p<0.05$, $p<0.01$ and $p<0.001$ leading to marking *, ** and *, respectively.**

The one way ANOVA yielded 26 significantly changed molecular features out of 341 molecular features. It was found that 11 molecular features accounted for monoacylglycerols (MG); MG(18:0), MG(16:0) and MG(14:0). The overall effect of the MGs was decreased levels in the plasma when exposed to BPA-0.25. Cholesterol was increased when rats were exposed to BPA-0.25. One metabolite found to be decreased already at BPA-0.025. This metabolite had an m/z of 359.149. It was not possible to identify a compound with this accurate mass on data base searches. Three of the molecular features match in identities with docosenamide, found both in the lipid and the polar fraction and for both a significantly decreased level in BPA-0.25; however as this metabolite is not normally present in mammalian plasma it was classified as an

impurity and the data was not further analyzed. In the phospholipid fraction no metabolites were found to be different. The rest of the significantly changed metabolites are shown in supplemental materials (supplemental materials)

Changes in female offspring:

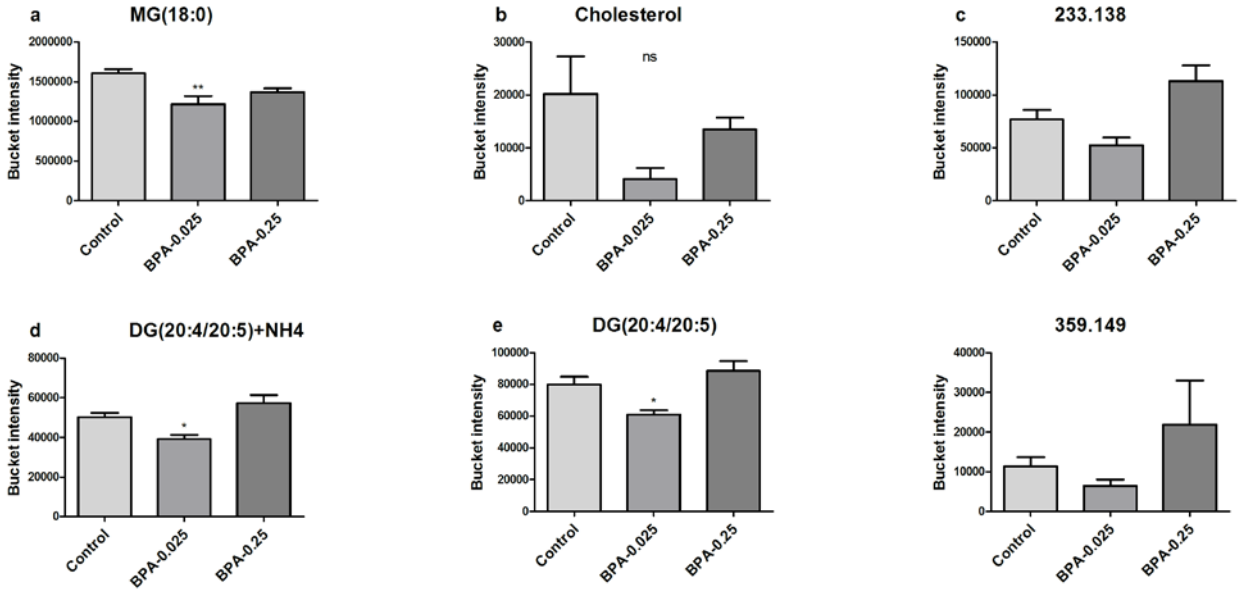

Figure 3 Metabolites from the plasma of the female offspring exposed to BPA at concentrations of 0.025 and 0.25 mg/kg bw/day. The results are shown as peak area ± SEM. As shown in the graphs there were significant decreases of MG(18:0) and DG(20:4/20:5), while no significant effect ass seen on cholesterol or the two unidentified metabolites with pseudo ion monoisotopic masses of 233.138 and 359.149. The criteria for statistical significance was p<0.05, p<0.01 and p<0.001 leading to markings of *, ** and *, respectively.**

It was found that 36 out of 671 molecular features were significantly different between control and BPA exposed animals for the female offspring. Several of these could not be identified as chromatographic peaks; ten molecular features were discarded due to this. Two diacylglycerols (DGs) and a MG were found to be decreased in the BPA-0.025 exposure group. Two other molecular features were found to be increased though it was impossible to establish their identities by use of accurate mass and MSMS patterns. Based on the increase in cholesterol from the dams, a targeted analysis using loss of water and Na adduct of cholesterol was conducted; however the concentration was not different between control and exposed animals.

Changes in male offspring animals:

Twenty-seven molecular features out of 347 were found to be significantly different between controls and exposed animals (See supplemental data). Of these 27 potential metabolites the identity of seven could be

established. In figure 4 five of the six identified metabolites are depicted, see Figure 4. There were increases in the concentrations of lyso-phosphotidylcholine (lyso-PC) and DG concentrations. For all the lyso-PC compound a “dose response” trend was observed, while for the neutral lipids and 359.149 there were only effects at BPA-0.25.

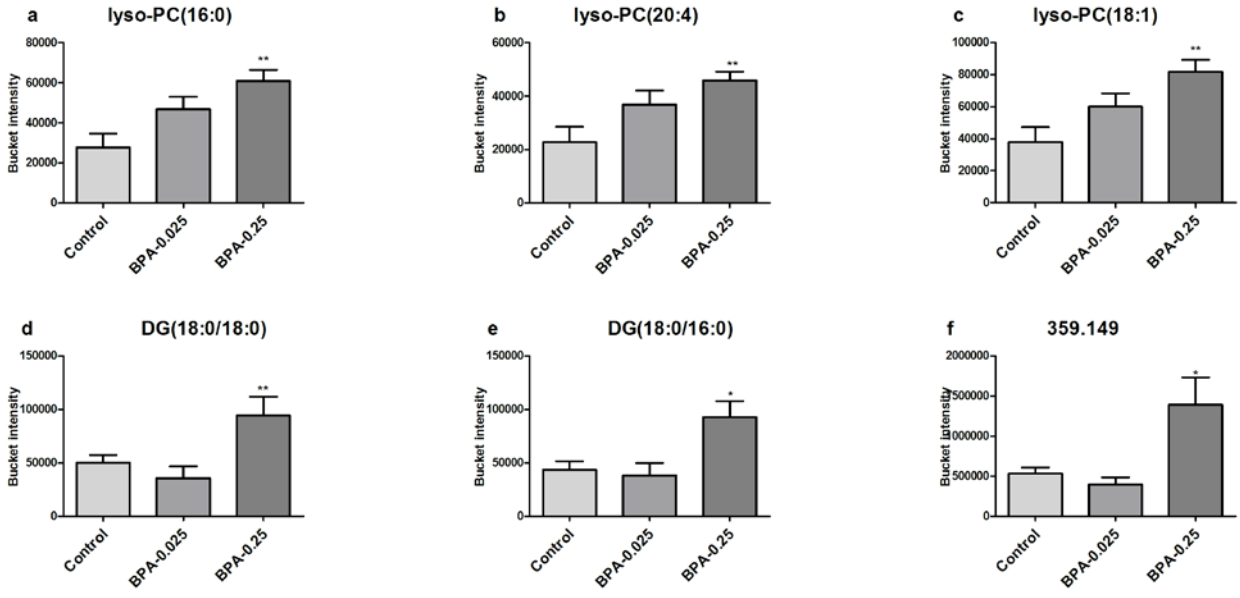

Figure 4 Significantly changed metabolites of male offspring exposed to BPA. The results are shown as peak area ± SEM. For the lyso-phosphotidylcholine (lyso-PC) (a-c) are increased when exposed to BPA-0.25. Furthermore was there an increase in two diacylglycerides and a compound with an accurate mass of 359.149. The criteria for statistical significance was p<0.05, p<0.01 and p<0.001 leading to markings of *, ** and *, respectively.**

Cholesterol was searched for by accurate mass searching for the sodium adduct and loss of water. However, no difference was found in the cholesterol plasma concentration in the male offspring. Furthermore, no effect was observed on the levels of MG(18:0) or MG(16:0) of the plasma from the male offspring (data not shown).

Discussion

In this study we found effects on the metabolome in dams and offspring of low doses of BPA administered prenatally. To evaluate the low dose toxic exposure metabolomics analysis was performed. The primary effect of BPA was evaluated by analysis of the plasma of the dam exposed during pregnancy and during the

lactation period. Furthermore, the plasma of female and male offspring was analyzed to evaluate whether an effect was seen after exposure through placenta and lactation.

The data from the dams displayed a significant change in the lipid metabolome. The small neutral lipids MG(18:0), MG(16:0) and MG(14:0) were significantly decreased when exposed to BPA-0.25 while cholesterol was found to be increased when exposed to BPA-0.25. This correlates with previously obtained results by Miyawaki *et al.* who have shown that cholesterol is increased in animals administered BPA orally in a concentration of 1 and 10 µg/kg bw/day (Miyawaki et al. 2007). However, this were not observed in neither male nor female offspring indicating that a direct exposure of BPA induces other effects than the secondary effect from the exposure via placenta and milk.

The metabolic pattern was significantly different between dams and offspring, though some of the metabolites were similar. The dams and the female offspring both suggest a lowering in MG(18:0). The male offspring furthermore have an increased concentration of lyso-PCs. These are involved in metabolism as well as used as signaling compounds in the body. The specific change of neutral lipids and lyso-PC is not fully understood in relation to their effects on health. Williams *et al.* (Williams et al. 2013) have shown that the lipid concentration is decreased in fruit flies when exposed to BPA and Boucher *et al.* (Boucher et al. 2014) have shown that TG accumulation is increased in adipose cells when exposed to BPA. Alonso-Magdalena *et al.* have proposed that administering BPA to mice increases the biosynthesis of insulin (Alonso-Magdalena et al. 2008). The increased level of insulin would increase the glucose uptake from the plasma thus inducing high energy state in the cell probably leading to an increase in biosynthesis of lipids.

One metabolite especially in focus in this study had a pseudo ion with accurate masse of 359.1493 ± 2 mDa. The metabolite is interesting as it was significantly decreased in the dam animals but significantly increased in both male and female offspring. The compound was searched for by accurate mass and a feature of Data Analysis 4.1 (Bruker Daltonics) suggested that the compound had the elemental composition $C_{20}H_{22}O_6$ but the exact identity of this compound could not be established.

The changes in the metabolomes of the dams and the offspring were not similar in the present investigation. However, some specific compounds were both found to be changed in dams and female offspring and dams and male offspring, respectively. It was not possible to detect one single mechanism of action for BPA from these data although it might involve changes of the lipid metabolism. At least such changes could be hypothesized to seriously impact the biochemistry and physiology of the body. A disruption in the non-polar part of the metabolome could explain some of the difference for this. Although it will be difficult to suggest a clear mechanism of primary and secondary exposure to BPA, we believe that

the data obtained from this study will support, via metabolic changes, which mechanism of actions BPA possess.

Conclusion

The effect of BPA is measurable even at concentration levels 20 and 200 times below no-observed-adverse-effect level. There is a difference in the metabolome between male and female rats. Furthermore, there is difference between dams administered to BPA directly and the offspring exposed through the dams (via placenta and milk in lactation period). One group of compounds namely the lyso-phospholipids, equally affected both dams and offspring as all were increased in concentration when exposed to BPA-0.25. The dam showed increased levels of cholesterol and a decreased level of monoacylglycerols while the male and female offspring showed an effect on diacylglycerol. One unidentified metabolite with m/z of 359.149 was of great interest as it was decreased in the dams while increased in male and female offspring. We believe that measuring the non-polar part of the metabolome has revealed new insight in the mechanism of action for bisphenol A, bringing us one step closer to understand how it affect animals as well as humans.

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Paper V **Metabolite profiling of urine from rats dosed with Selenium nanoparticles or Selenium ions**

Manuscript in progress

Aim: In the present investigation we utilized urine samples from rats administered with selenium in two different physical formulations (as nanoparticles and as sodium selenite) to assess the usefulness of metabolite pattern determination for comparing effects on mechanism of action of different chemicals.

Results: We found that when evaluating all significantly changed metabolites, nanoparticles and sodium selenite induced similar patterns of regulation. In the urine 13 metabolites were significantly different after exposure to selenium. Moreover we estimated the potencies to be similar. This suggests that selenium nanoparticles are completely dissolved into ions in the gastrointestinal system of the rat, and exhibit a bioavailability and mechanism of action similar to that of selenium ions.

Conclusion: We found that metabolite profiling in urine can be used to compare different chemical congeners in terms of similarities and differences in toxicological mechanisms of action.

Comparison of biological effect profiles of chemical congeners using metabolite pattern determination the example of selenium nanoparticles and sodium selenite

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ABSTRACT

Introduction: In pharmacology and toxicology, there is often a need to determine differences in the biological effect profiles of chemical congeners. This is important in order to assess whether alterations in the chemical composition or formulation have an impact on the biological effects of the substance. Using metabolite pattern determination, differences in the effect profile can be assessed by determining whether there are differences among the sets of metabolites altered by the various congeners. **Methods:** We utilized urine samples from rats administered selenium in two different physical formulations (as nanoparticles or as sodium selenite) to assess the utility of metabolite pattern determination in comparisons of different congeners. **Results:** Evaluations of all significantly changed putative metabolites showed that selenium nanoparticles and sodium selenite induced similar patterns of regulation. Moreover, we estimated the bioavailability to be in a similar range with a tendency to a higher bioavailability of selenite compared to selenium nanoparticles. **Discussion:** metabolite pattern determination demonstrated that selenium nanoparticles exhibit an effect profile similar to that of selenium ions. This suggests that the effects of nanoparticulate selenium can be attributed to ions released from its surface.

1. Introduction

In pharmacology and toxicology, there is often a need to determine differences in the effect profiles of chemical congeners. This is important in order to provide a picture of whether a (physico) chemical change or alterations in formulation alters the effect of each congeners on the mammalian body. This may be in general but when specific mechanisms are known and measured a picture of efficacy profile versus toxicity profile as well as information on bioavailability may be assessed specifically. Examples of such congeners are different physico-chemical states of elements (*e.g.*, nanoparticles *vs.* ions) or different functional groups applied to principal structures in drug research. Metabolite pattern determination draws on the concept of metabolite profiling (metabolomics) to enable the concomitant measurements of a large number of chromatogram peaks containing potential metabolites (Robertson, Watkins, & Reily, 2011). Using metabolite pattern determination on a body compartment such as urine or blood plasma, differences in the biological effects can be assessed by comparing the sets of metabolites that are altered by each congener. This provides a combined picture of pharmaco- and toxicodynaimcs as well as –kinetics .

Selenium (Se) is an element that, in trace amounts, is essential for humans. Se is incorporated as selenocysteine, and selenium is necessary for the synthesis and catalytic function of selenoproteins such as peroxidases and reductases (Levander, 1982). At high doses, Se becomes toxic, exhibiting effects such as emaciation and weight loss (Nogueira & Rocha, 2011). To provide more effective Se dosing regimens, nanoparticle formulations have been developed. The concept is that the Se nanoparticles provide a slow release of Se ions, thereby reducing acute toxicity. Several studies have demonstrated a lower toxic potency of Se nanoparticles than of ionic Se, suggesting that to some extent, Se nanoparticles remain intact in the gastrointestinal tract (Benko et al., 2012; Jia, Li, & Chen, 2005; J. S. Zhang, Gao, Zhang, & Bao, 2001; J. Zhang, Wang, Yan, & Zhang, 2005). It has been shown that smaller Se nanoparticles are more toxic than larger particles. This finding suggests that the rate of release of Se ions may depend on the nanoparticle size and thus on the surface area per unit weight (Peng, Zhang, Liu, & Taylor, 2007). Whether Se nanoparticles exhibit unexpected effects that cannot be attributed to Se ions but rather to the physical form of the nanoparticles themselves remains unknown. In the present investigation, we used equimolar amounts of Se formulated in two different ways (nanoparticles *vs.* ions), to study the utility of metabolite pattern determination for the differentiation between the biological effects of chemical congeners. We used LC-MS to analyze urine samples obtained after 14 days of oral administration

of Se nanoparticles (19 nm in mean diameter) or sodium selenite in equimolar doses of 0.05 and 0.5 mg Se/kg bw/day. A maximal safe dose in humans is 5 µg/kg bw thus these doses reflect 10 and 100 fold that dose. The affected metabolites were determined using the MetaboAnalyst web-tool (Xia, Psychogios, Young, & Wishart, 2009), and the curves obtained were assessed for equal effect patterns to compare differences in biological effect profiles for the different congeners.

2. Materials and methods

2.1. Nanoparticles

Se nanoparticles with a mean diameter of 19 nm (ranging in size from 10 to 80 nm) were produced, stored and characterized as previously described (Loeschner et al., 2014; J. S. Zhang et al., 2001). Briefly, images to assess the size and shape of the Se nanoparticles were generated by transmission electron microscopy (TEM) (Figure 1) using a TEM Philips CM100 instrument (FEI, Eindhoven, The Netherlands) at 80 kV accelerating voltage (Loeschner et al., 2014).

2.2. Animal study

Urine samples were obtained from a previously reported Se bio-distribution study (Loeschner et al., 2014). Briefly, four-week-old, specific pathogen-free (SPF) female Wistar rats were obtained from Taconic M&B (Lille Skensved, Denmark). The rats were allowed to acclimatize for one week. The rats were housed in pairs with a 12:12-h light/dark cycle with the lights on from 7 a.m. to 7 p.m. The room temperature was 22 ± 1 ^{o}C, and the relative humidity was 55 % ± 5 %. The rats were given ad libitum access to a standard diet (Prod. no.1324, Altromin International, Lage, Germany) and citric acid acidified tap water. The animals were randomized by weight. The test substances were administered by oral gavage in a volume of 10 mL/kg bw once a day for 14 days. The treatment groups were as follows: 1. Vehicle-BSA (bovine serum albumin 4 g/L) control (n=8); 2. Se nanoparticles 0.05 mg Se/kg bw/day stabilized with BSA (4 g/L) (n=6); 3. Se nanoparticles 0.5 mg Se/kg bw/day (n=6) stabilized with BSA (4 g/L); 4. Vehicle-H_2O (n=8); 5. Sodium selenite

0.05 mg Se/kg bw/day (n=8); and 6. Sodium selenite 0.5 mg Se/kg bw/day (n=8). No BSA was added to groups 4, 5 and 6; thus, group 4 acted as the control group for the sodium selenite groups. Sodium selenite (Na_2SeO_3) was obtained from Sigma-Aldrich (Copenhagen, Denmark). On experimental day 14, the rats were placed individually in metabolism cages for 24 hours for the collection of urine. During the 24-hour period, the urine samples were frozen by collection on dry-ice. Subsequently, the samples were stored at -80 °C. In the metabolism cages, the rats had access to drinking water but not to feed. The animal study was conducted under conditions approved by the Danish Animal Experiments Inspectorate and the in-house Animal Welfare Committee.

2.3. HPLC/MS metabolite profiling analysis

Metabolite profiling of the urine was performed as previously described (Hadrup et al., 2012). In brief, the urine samples were precipitated with two volumes of methanol and centrifuged (10 min at 10,000 x g). The supernatants were collected and analyzed by HPLC coupled to a qTOF-MS. Sample injection volumes were normalized to the creatinine concentration of the urine to adjust for differences in diuresis. The metabolites were separated on an Avensis Express C8, 2.7 µm, 100 x 2.1 mm column (Supelco, Bellefonte, PA, USA, product no. 53832-U). The metabolites were next detected by use of a Bruker microTOFq time-of-flight mass spectrometer equipped with an electrospray ion source (Bruker Daltonics, Bremen, Germany). The samples were analyzed in both positive and negative ionization modes. The data obtained are reported as the mass-to-charge (m/z) ratios and HPLC retention times of the metabolites. These are given in the format of xxxx.xxx Da, yyy.y s (seconds).

The analyses of the chromatograms were conducted using the Profile Analysis 2.1 software package (Bruker Daltonics, Bremen, Germany). Data buckets were constructed using a time window from 60 to 720 s with an m/z ratio range of 100 to 700 using the “find molecular feature”

algorithm including time alignment. The noise was reduced using R (R Core Team, 2012) by removing peaks that were present in < 50% of the samples of each treatment groups and had peak intensities of ≤ 3000 counts per s (cps). The raw intensity data were next transferred to the online MetaboAnalyst server (Xia et al., 2009). The data were normalized as the sums, and Pareto-scaling was performed. Partial least squares (PLS) discriminant analysis was performed for all datasets collectively. ANOVA was applied to the BSA-vehicle *vs.* Se nanoparticle groups and separately to the H_2O vehicle *vs.* sodium selenite groups. Discriminatory metabolites were selected based on a false discovery rate-adjusted p value of 0.05. The corresponding raw data for these metabolites were then transferred to Graph Pad Prism to establish curves and test for normal distributions. Normality was tested using the Kolmogorov Smirnov test (with Dallal-Wilkinson-Lillefor p-value). The data (BSA vehicle *vs.* Se nanoparticle groups and H_2O vehicle *vs.* selenite groups) were then evaluated again by ANOVA or by Kruskal-Wallis depending on the presence or absence of normal distribution. Dunnett's and Dunn's post tests were applied to determine the effects of single treatment groups compared to their respective control groups. All data on the graphs are presented as the mean, and the error bars represent SEM. A false discovery rate-corrected p-value of less than 0.05 was considered significant.

The metabolites were subjected to identification to provide a level of certainty that chromatographic peaks represented metabolites and to provide a picture of the nature of the effects induced by the selenium congeners. For the identification of metabolites, The Human Metabolome Database (Wishart et al., 2013) was searched using the accurate masses of the metabolites. The presence of adducts or fragments (*e.g.*, plus Na^+ or minus H_2O) at identical HPLC retention times was taken into account.

3. Results

The three-dimensional graphical presentation of the results of the PLS analyses provides a picture of the overall differences among the treatment groups (Fig. 2). In the positive mode, there were clear differences in the spatial locations of all groups, reflecting dose-response effects as indicated by the position of the 0.05 mg Se group between the vehicle and the 0.5 mg Se groups. There was a clear vehicle-induced difference in the spacing of samples in the PLS analyses as indicated by the different position of the BSA-vehicle samples compared to the H_2O-vehicle samples (Fig. 2). In the negative mode, the picture was similar to that seen for the positive mode, although less clear (Fig. 2).

Concomitant analyses of all groups using the MetaboAnalyst online tool indicated statistically significant changes in eight peaks in the negative mode and 11 in the positive mode (Tables 1 and 2). Some of these were adducts of others (see Tables 1 and 2), and after accounting for these, six metabolites were significantly changed in the negative mode and seven in the positive mode (Figure 3). Several metabolites showed dose-response relationships as suggested by the graphs. However, application of the post-test identified statistically significant effects only for the 0.5 mg Se/kg bw/day groups. Overall, the Se nanoparticles and sodium selenite induced similar patterns of regulation. Exceptions were metabolites 316.1229 Da, 233.9 s in negative mode and 367.2448 Da, 236.8 s in positive mode (see Tables 1 and 2 for the possible identities of these metabolites). The levels of the effects (intensity counts) likewise seemed similar, although the number of enhanced metabolites in the urine of the sodium selenite-administered animals was larger than in the Se nanoparticle-administered animals. The exact mass of discriminatory metabolites (± 5 mDa) was used as a search parameter in The Human Metabolome Database (Wishart et al., 2013). The putative identities of the metabolites are presented in Tables 1 and 2.

4. Discussion

The purpose of this investigation was to evaluate the utility of metabolite pattern determination in the comparison of chemical congeners with respect to similarities in the biological effect profiles. For this study, we utilized urine from rats administered two chemical forms of Se, specifically elemental Se nanoparticles or sodium selenite. The PLS analyses indicated effects of both formulations at the low (0.05) and the higher (0.5 mg/kg bw/day) Se doses (Figure 2). Among the 13 metabolites found to be significantly changed, 11 displayed similar patterns of metabolite regulation for both Se formulations (Figure 3). Assuming that urine reflects the excreted end products of a high proportion of the body's metabolic processes, there is a strong indication of similar biological effects of the two different formulations. However, to providing details on whether these similarities were due to equal (toxicological) mechanisms of action it would be necessary to know if the altered metabolites reflected the toxicological mechanisms of selenium. It has previously been shown that selenium induces liver and bone toxicities (HASEGAWA, ZHANG 2005, ISHIKAWA, TURAN 2000, YAMAGUCHI 2000). But information on differences in metabolite patterns are lacking.

The putative identification of the metabolites was attempted by searching The Human Metabolome Database using the exact mass of the ions. However, more than one candidate identity was often possible for each single metabolite. This was due to similar elemental composition; e.g., for one ion, we found 12 different possible isoforms of diacylglycerol with exactly the same molecular mass.

This hampered the determination of specific mechanisms of action for Se. However, overall, there were changes in fatty acids and amino acids, suggesting that there is a general response on metabolism following the administration of Se in the doses employed.

Thus the metabolite differences could not be tied directly to a mechanism of action of selenium. However, such information may very well be the case for other chemical congeners e.g. when a leading pharmaceutical structure has already been profiled for its mechanism of action. And when this is the case a difference in some metabolites (e.g. those associated with adverse effects of the leading structure) but not others (e.g. those associated with the wanted pharmaceutical effect) can provide picture on advantages and disadavantages of inducing a certain chemical change or formulation change to a leading structure.

Rather than giving us a specific knowledge on equal mechanisms of action of the similarity of the two selenium formulations give an indication of a similar general response in terms of a combination of such effects in the body as mechanism of action both on the intended target, different metabolism pathways, different distribution, differences in excretion. We suggest that the data could be used to estimate the biovaialbility of the two Se formulations to be in a similar range, with a tendency to a bioavailability of selenite compared to Se nanoparticles. This tendency is given by eight metabolites for which there was a significant effect of sodium selenite but only a non-significant tendency for Se nanoparticles (Fig. 3). This finding could be due to a high degree of dissolution of the Se nanoparticles into ions, probably occurring in the gastro-intestinal tract. Others have found that for some toxicity endpoints, selenite was more potent than Se nanoparticles in mice and rats (Benko et al., 2012; Jia et al., 2005; J. S. Zhang et al., 2001; J. Zhang et al., 2005). However, in spite of this tendency, our previous finding that the high doses of both forms of Se were equally available for incorporation into selenoprotein P supports the hypothesis that the bioavailability was in a similar range (Loeschner et al., 2014). Moreover, Kojouri and co-workers

found similar effects of Se nanoparticles and sodium selenite on iron, transferrin and on neutrophils in sheep (Kojouri, Jahanabadi, Shakibaie, Ahadi, & Shahverdi, 2012; Kojouri, Sadeghian, Mohebbi, & Mokhber Dezfouli, 2012).

Pattern recognition approaches in chemometrics and metabolite profiling have been described and employed previously by others (el-Deredy, 1997; Robertson et al., 2011; Robertson, 2005). These approaches have, for example, been used to identify specific metabolite patterns for specific toxicities without knowing the identity of the specific metabolites (Anthony, Rose, Nicholson, & Lindon, 1995; Holmes et al., 1994, 1998; Lenz, Bright, Knight, Wilson, & Major, 2004). However, there are arguments against this approach. As reviewed by Robertson (Robertson et al., 2011; Robertson, 2005), for example, effects from the primary toxicity of a compound and concomitant indirect toxicities may overlay and obscure the picture of specific organ toxicities. In addition, the metabolite profile may reflect a minor toxicological target, and therefore, concomitantly activated major targets may be missed. Robertson noted that it could therefore be advisable to focus on determining the exact identities of metabolites, to establish biomarkers that are understood in terms of whether they reflect either effects or mechanisms (Robertson et al., 2011). However, in the use proposed here, metabolite patterns are highly useful for assessing differences in biological effects between different chemical congeners, even in the absence of confirmed identification of the metabolites. Other potential uses are the determination of differences between a novel substance and a known standard or the determination of whether impurities have biological effects. For these applications, different treatment groups are tested simultaneously to minimize the influence of environmental factors such as different stress levels between different animal facilities or differences in feed batches, etc., on the results of the study.

In conclusion, we used a metabolite pattern determination approach to compare the biological effect profiles of Se nanoparticles and sodium selenite. Based on our findings of similar patterns of

metabolites in the urine, we conclude that it is highly likely that the two formulations of Se have similar biological effects. Moreover, comparisons of the intensities of the effects indicated that the formulations exhibited similar bioavailability. This suggests that the effects of nanoparticulate Se can be attributed to ions released from its surface. We propose that determination of metabolite patterns is a promising approach in pharmacological and toxicological studies in which different chemical congeners are compared to determine differences in their effect profiles, including differences in their mechanisms of action, metabolism and bioavailability.

Figures

Figure 1.

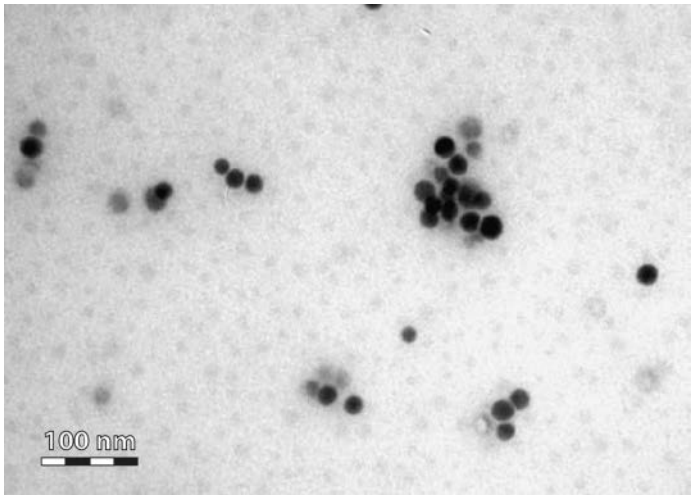


Figure 1. TEM micrograph of the administered Se nanoparticles

Figure 2.

Fig 2A

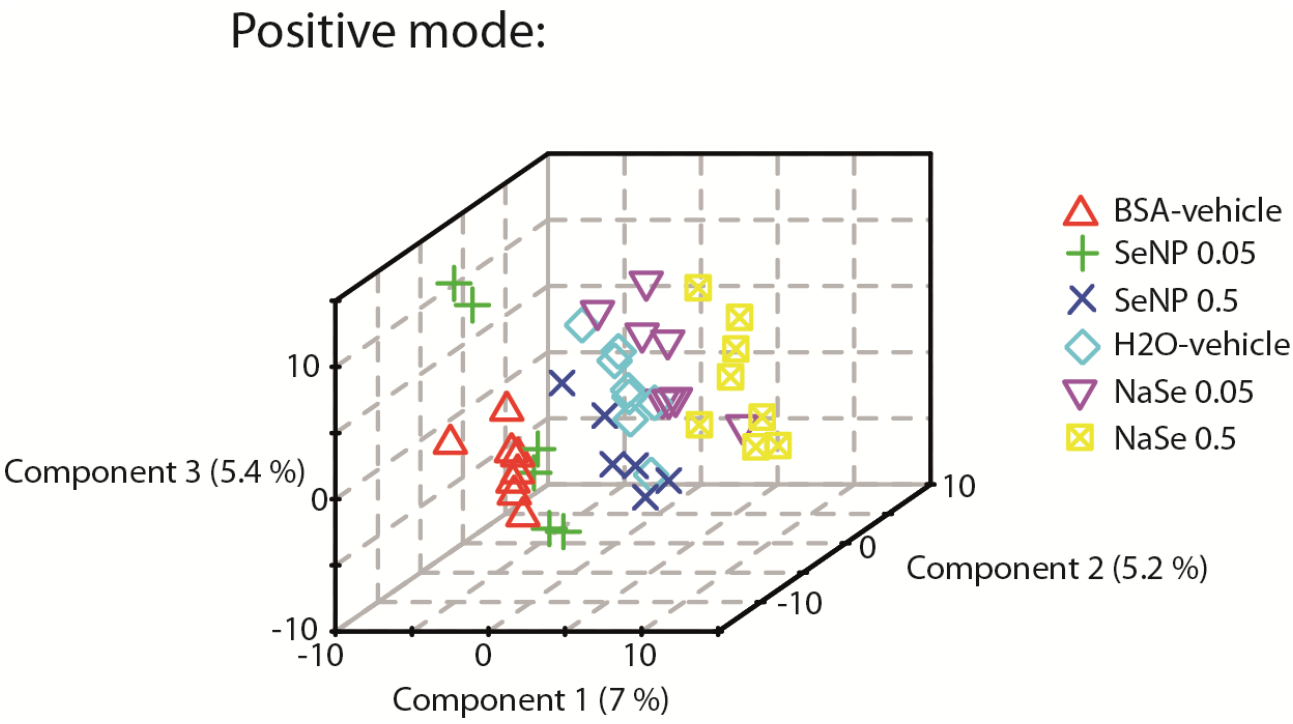


Fig 2B:

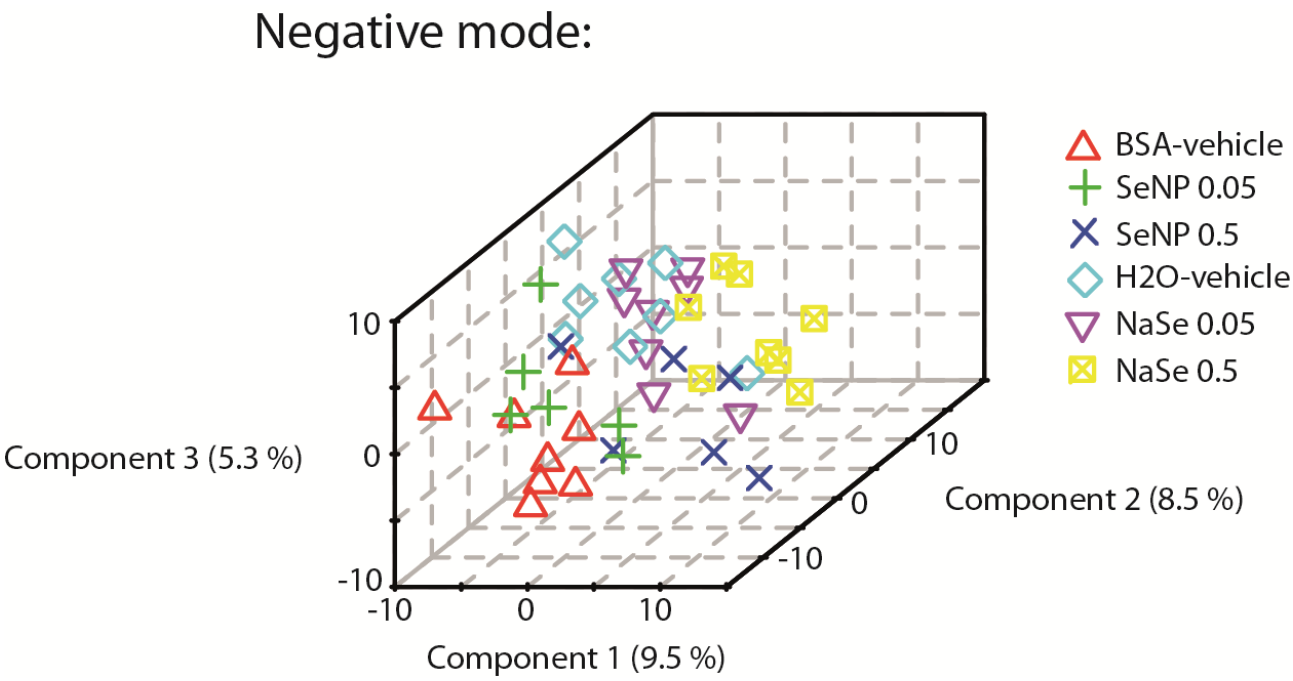
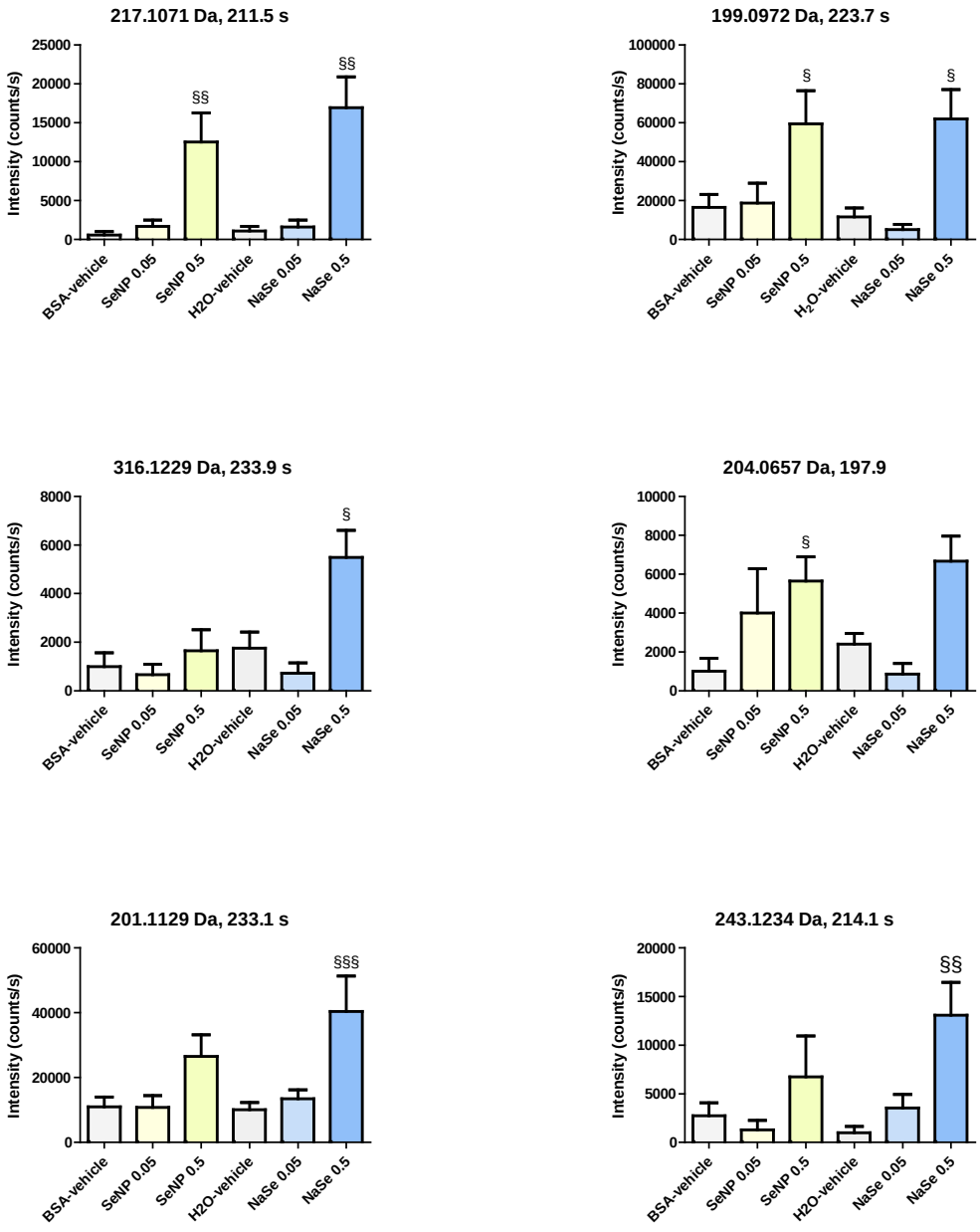


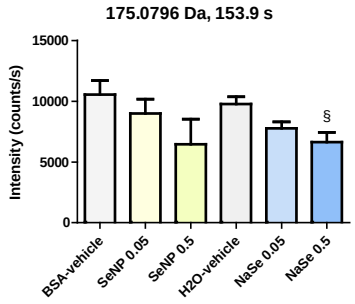
Figure 2. PLS analyses of urine from rats administered Se nanoparticles or sodium selenite. SeNP designates Se nanoparticles, and NaSe designates sodium selenite. The components designate principal components 1-3 of the PLS analyses.

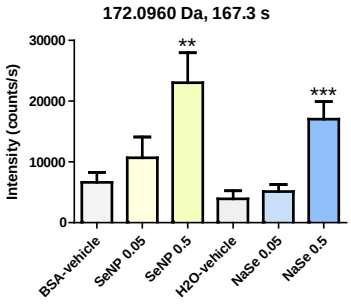
Figure 3.

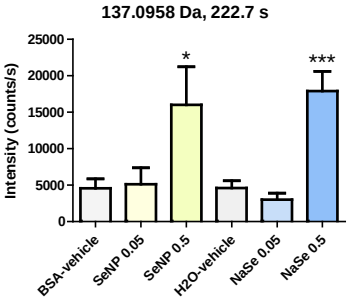
A) Negative mode:

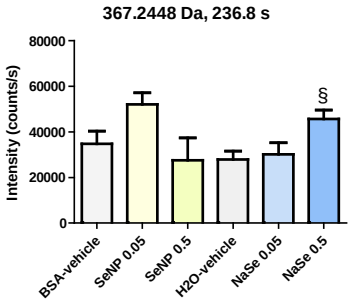


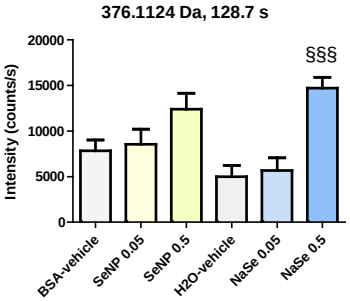
B) Positive mode

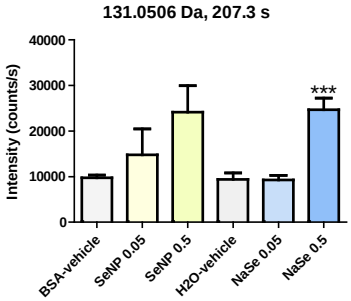












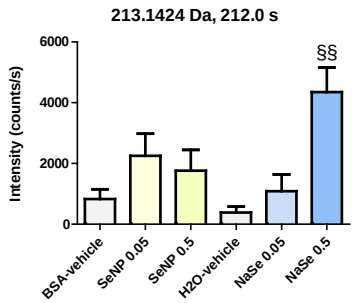


Figure 3. Bar graphs of individual metabolites determined by LC-MS in A) negative ion mode or B) positive ion mode and indicated by their accurate masses. SeNP designates Se nanoparticles, and NaSe designates sodium selenite. The data are the mean values, and the bars indicate SEM. The data were statistically tested as BSA-vehicle *vs.* Se nanoparticles at 0.05 and 0.5 mg Se/kg bw/day and as H_2O-vehicle *vs.* sodium selenite at 0.05 and 0.5 mg Se/kg bw/day. The tests were one-way ANOVA with Dunnett's post-test for normally distributed data (* designates $p < 0.05$, **$p<0.01$ and ***$p<0.001$), or with Kruskal-Wallis with Dunn's post-test for data that were not normally distributed (§ designates $p < 0.05$, $^{\S\S}p<0.01$ and $^{\S\S\S}p<0.001$).

Table 1. Possible identities of metabolites measured in negative mode

Metabolite m/z (Da)	HMDB m/z (Da)	m/z error (Da)	Ret. Time (s)	Possible identity
217.1071	217.1082	0.0011	211.5	2-Hydroxydecanedioic acid / 3-Hydroxydecanedioic acid *[M-H]*
199.0972	199.0976	0.0004	223.7	cis-4-Decenedioic acid / cis-5-Decenedioic acid *[M-H]*
316.1229	316.1303	0.0074	233.9	Tryptophyl-Hydroxyproline / Hydroxyprolyl-Tryptophan *[M-H]*
204.0657	204.0666	0.0009	197.9	Cinnamoylglycine / Indolelactic acid / 3-Indolehydracrylic acid / 5-Methoxyindoleacetate *[M-H]*
201.1129	201.1132	0.0003	233.1	Sebacic acid / Heptylmalonic acid / 3-Methylazelaic acid *[M-H]*
160.0757	161.0688	0.0141	197.9	Possible fragment of 204.0657 *[M-CH_2O_2]*
181.0873	n/a	n/a	223.7	cis-4-Decenedioic acid / cis-5-Decenedioic acid *[M-H2O]*
243.1234	243.1167	0.0067	214.1	Isoleucyl-Methionine / Methionyl-Isoleucine *[M-H20-H]*

Legend: Potential metabolites identified by comparing m/z ratios of metabolites with m/z ratios obtained from the human metabolome (HMDB) database. Ret. Time: Retention time.

Table 2. Possible identities of metabolites measured in positive mode

Metabolite (m/z)	HMDB MW (m/z)	Error (m/z)	Ret. Time (s)	Possible identity
175.0796	175.0713	0.0083	153.9	N-Acetylasparagine / Formiminoglutamic acid $[M+H]^+$
192.1042	192.0979	0.0063	153.9	N-Acetylasparagine / Formiminoglutamic acid $[+NH4^+]$
172.0960	n/a	n/a	167.3	No candidates
137.0958	137.0921	0.0037	222.7	L-Threonine / L-Allothreonine $[M+NH4^+]$
201.1098	201.1121	0.0023	219.3	cis-4-Decenedioic acid / cis-5-Decenedioic acid $[M+H]^+$
203.1268	203.1277	0.0009	233.8	Sebacic acid / Heptylmalonic acid / 3-Methylazelaic acid $[M+H]^+$
367.2448	367.2426	0.0022	236.8	12 different diacylglycerols $[M+Na]^+$
165.0912	n/a	n/a	222.1	No candidates but fits with 137.0958 plus CO $[M+H]^+$
376.1124	n/a	n/a	128.7 s	No candidates
131.0506	131.0503	0.0002	207.3	N-a-Acetyl-L-arginine *[M+2Na]*
	131.0514	0.0008		Glutamyl-Asparagine / Asparaginyl-Glutamate *[+2H]*
213.1424	213.1485	0.006	212.0	Six different bile acids $[M+H]^+$

Legend: Potential metabolites identified by comparing m/z ratios of metabolites with m/z ratios obtained from the human metabolome (HMDB) database. Ret. Time: Retention time.

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Author contributions

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